CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 51/10, 49/00, 39/395, C07K 16/30, C12N 5/12, G01N 33/53

A1

(11) International Publication Number: WO 96/40295

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09819

(22) International Filing Date: 7 June 1996 (07.06.96)

(30) Priority Data:
08/478,190 7 June 1995 (07.06.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/478,190 (CIP)
Filed on 7 June 1995 (07.06.95)

(71) Applicant *(for all designated States except US)*: SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LLOYD, Kenneth, O. [US/US]; 425 Henry Hudson Parkway West, Bronx, NY 10471 (US). FEDERICI, Mark, G. [US/US]; 19 Glencross Road, West Milford, NJ 07480 (US). DIVGI, Chaitanya, R. [IN/US]; 401 East 89th Street #16A, New York, NY 10128 (US). KOSTAKOGLU, Lale [TR/TR]; Turan Gunes Cad. No. 63/39, Cankaya 06550, Ankara (TR).

(74) Agent: WHITE, John, P.; Cooper & Dunham L.L.P., 1185 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AU, CA, IL, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: MONOCLONAL ANTIBODY FRAGMENT TO HUMAN OVARIAN CANCERS

(57) Abstract

The biodistribution and pharmacokinetics of intact and $F(ab')_2$ fragments of radiolabeled (^{125}I) monoclonal antibody (Mab) MX-35 and a control mAB were compared in nude mice bearing xenografts of OVCAR-3 human epithelial ovarian cancer. The highest tumor to blood ratio was 12.8 for the $F(ab')_2$ fragment and 1.9 for intact Mab MX-35. Maximum tumor to normal tissue ratios were 64 for fragment and 12.3 for intact antibody. The maximum percent injected dose per gm (%IDg^{-1}) of Mab in tumor was 10.4 % for $F(ab')_2$ fragments and 1.6 % for intact antibody when administered intravenously and 8.2 % for $F(ab')_2$ fragments and 2.3 % for intact MX-35 when injected intraperitoneally. The finding of higher percentage ID g^{-1} for the fragments is unexpected and is in contrast to other published studies.

* (Referred to in PCT Gazette No. 10/1997, Section II)

MONOCLONAL ANTIBODY FRAGMENT TO HUMAN OVARIAN CANCERS

This invention was partially made with funds provided by the National Cancer Institute under grants CA-26184 and CA-08748. Accordingly, the United States Government has certain rights in this invention.

This invention relates to a method for the production of monoclonal antibodies (monoclonal antibodies) to restrictive antigenic human cell components especially in human ovarian tissues. Such monoclonal antibodies have use in cancer diagnosis and therapy, as well as other cell disorders.

Background

Conventional antisera, produced by immunizing animals with tumor cells or other antigens, contain a myriad of different antibodies differing in their specificity and properties. In 1975 Köhler and Milstein (Nature, 256:495) introduced a procedure which leads to the production of quantities of antibodies of precise and reproducible specificity. The Köhler-Milstein procedure involves the

fusion of spleen cells (from an immunized animal) with an immortal myeloma cell line. By antibody testing of the fused cells (hybridomas), clones of the hybridomas are selected that produce antibody of the desired specificity. Each clone continues to produce only that one antibody, monoclonal antibody (monoclonal antibody). As hybridoma cells can be cultured indefinitely (or stored frozen in liquid nitrogen), a constant, adequate supply of antibody with uniform characteristics is assured.

Antibodies are proteins that have the ability to combined with and recognize other molecules, known as antigens. Monoclonal antibodies are no different from other antibodies except that they are very uniform in their properties and recognize only one antigen or a portion of an antigen known as a determinant.

In the case of cells, the determinant recognized is an antigen on or in the cell which reacts with the antibody. It is through these cell antigens that a particular antibody recognizes, i.e. reacts with, a particular kind of cell. Thus the cell antigens are markers by which the cell is identified.

These antigenic markers may be used to observe the normal process of cell differentiation and to locate abnormalities within a given cell system. The process of differentiation is accompanied by changes in the cell surface antigenic phenotype, and antigens that distinguish cells belonging to distinct differentiation lineages or distinguish cells at different phases in the same differentiation lineage may be observed if the correct antibody is available.

The preparation of hybridoma cell lines can be successful or not depending on such experimental factors as nature of the innoculant, cell growth conditions, hybridization conditions etc. Thus it is not always possible to predict successful hybridoma preparation of one cell line although success may have been achieved with another cell line. But it is often true that selected monoclonal antibody may be representative of a class of monoclonal antibody raised by a particular immunogen. Members of that class share similar characteristics, reacting with the same cell antigen. Thus the invention includes hybridoma cell lines and monoclonal antibody with like or similar characteristics.

Progress in defining cell surface antigens is of great importance in differentiation and disease as markers for normal and diseased cells, thereby furthering diagnosis and treatment. Thus work on melanocytes was made possible by the recently discovered technique of culturing melanocytes from normal skin (Eisinger, et al., Proc. Nat'l. Acad. Sci. USA, 79 2018 (March 1982). This method provides a renewable source of proliferating cells for the analysis of melanocyte differentiation antigens. Likewise, a large number of cell lines derived from melanomas have now been established and these have facilitated the analysis of melanoma surface antigens. The advent of monoclonal antibodies has greatly accelerated knowledge about the surface antigens of malignant melanoma. cell markers on both melanomas and melanocytes have been identified. A panel of typing monoclonal antibodies has been selected which recognizes differentiation antigen characteristics at each stage of development in both melanocytes and melanomas. These differentiation antigens may be used to classify melanocytes and melanomas and to group them into characteristic sub-sets. [Dippold et al. Proc. Nat'l.Acad. Sci. U.S.A. 77, 6114 (1980) and Houghton, et al. J. Exp. Med. 156, 1755 (1982)]. Immunoassay of melanocytes and melanoma cells within sub-sets is thus made possible.

Initial recognition of differentiation antigens came about through analysis of surface antigens of T-cell leukemias of the mouse and the description of the TL, Thy-1, and Lyt series of antigens. (Old, Lloyd J., Cancer Research, 41, 361-375, February 1981) The analysis of these T-cell differentiation antigens was greatly simplified by the availability of normal T cells and B cells of mouse and man. (See Patents #4,361,549-559; #4,364,932-37 and #4,363,799 concerning monoclonal antibody to Human T-cell antigens).

The existence of human leukemia specific antigens has been suggested by studies using heterologous antibodies developed by immunization with human leukemic cells [Greaves, M.F. et al. Clin. Immunol. and Immunopathol 4:67, (1975); Minowada, J., et al. J. Nat'l. Cancer Insti. 60:1269, (1978); Tanigaki, N., et al. J. Immunol. 123:2906, (1979)] or by using autologous antisera obtained from patients with leukemia [Garret, T.J., et al., Proc. Nat'l. Acad. Sci. USA 74:4587, (1977); Naito, K., et al., Proc. Nat'l. Acad. Sci. USA, 80: 2341, (1983)]. The common acute lymphoblastic leukemia antigen (CALLA) which is present on leukemia cells from many patients with non-T, non-B, acute lymphoblastic leukemia (N-ALL), some chronic myelocytic leukemias (CML) in blast crisis and a few acute

T-lymphoblastic leukemias (T-ALL) was originally described using conventional rabbit heteroantisera [Greaves, M.F. et al. Supra].

By the autologous typing technique [Garret, T.J., et al. Supra; Naito, K., et al. Supra 1983; Old, L.J. Cancer Res. 41:361, (1981)], antibodies uniquely reacting with ALL cells were found in sera obtained from patients with ALL, and seemed to recognize very similar antigens to CALLA (Garret, T.J., et al. Supra; Naito, K., et al. Supra). Another leukemia associated antigen detected by heterologous antisera is the human thymus leukemia (TL)-like antigen, which is present on thymocytes as well as leukemia cells (Tanigaki, N. et al. Supra). This antigen, is therefore, a normal differentiation antigen which is composed of a heavy chain (MW 44,000-49,000) and light chain (MW 12,000-14,000) similar to the class I HLA antigens (Tanigaki, N., et al. Supra). These investigations have, however, been hampered by the need for vigorous absorptions with normal tissues as well as the relatively small quantity and low titer of the antisera.

In vitro production of monoclonal antibodies by the technique of Köhler and Milstein, Supra has provided a better system for the identification and detection of

leukemia specific antigens. A panel of monoclonal antibodies detecting cell surface antigens of human peripheral blood lymphocytes and their precursor cells have been investigated in detail [Reinherz, E.L., et al. Proc. Nat'l. Acad. Sci. USA 77:1588, (1980)]. While monoclonal antibodies detecting antigens characteristic for different lymphocyte lineages can be used for classification of human lymphocytic leukemia [Schroff, R.W., et al. Blood 59: 207, (1982)], such antibodies have only limited therapeutic applications. Monoclonal antibodies detecting human leukemia associated antigens have also been produced. These include several antibodies detecting the human equivalents of the murine TL antigens. One TL-like antigen is recognized by NA1/34 [McMichael, A.J., et al. Eur. J. Immunol. 9:205, (1979)], OKT6 (Reinherz, E.L., et al. Supra) and Leu 6 (R. Evans, personal communication). A second TL-like antigen is recognized by M241 (Knowles, R.W., et al. Eur. J. Immunol. 12: 676,1982). Monoclonal antibodies with specificities for common acute lymphoblastic leukemia antigens J-5 (Ritz, J., et al. Nature 283:583, 1980), NL-1 and NL-22 (Ueda, R., et al. Proc. Nat'l. Acad. Sci. USA 79:4386, 1982) have also been produced. Recently, Deng, C-T, et al. Lancet. i:10, 1982) reported a complement fixing monoclonal antibody (CALLA-2) which reacts with most cultured human T-ALL cell lines and also reacts with most fresh T-ALL cells.

Mouse monoclonal antibodies to human tumor cell surface antigens have been produced in many laboratories (Lloyd, K.O. (1983) In: Basic and Clinical Tumor Immunology, Vol. 1 (R.B. Herberman, Ed.), Nijhoff, The Hague (in press)). The intention of these studies often has been to identify tumor-associated antigens that could be useful in tumor therapy or diagnosis. An inherent difficulty in this approach is the diversity of antigens on the cell surface. Although it has been possible to identify some antigens with a very restricted distribution, antibodies to antigens that elicit very weak immune responses may be missed due to their scarcity. These restricted antigens may be quite difficult to identify. Also, immunization with a complex mixture of antigens such as tumor cells may suppress the antibody response to relatively less immunogenic molecules, in a manner resembling antigenic competition (Taussig, M.J. (1973). Curr. Top. Micro. Immuno. 60:125). Thus production of monoclonal antibody to restricted cell sites is an especially difficult task. The present invention provide cancer diagnosis and therapy and overcome problems heretofor encountered in the prior art with respect to ovarian and endometrial human cell antigens.

The production and characterization of mouse monoclonal antibodies (mAbs) to human tumor cells has recently been an active area of research in recent years. This effort was stimulated by many earlier immunological studies which suggested that existence of human tumor-specific antigens, but such evidence has not been conclusive, and has not been consistently reproduced in other laboratories, making the status of such antigens uncertain (reviewed in Old, L.J. Cancer Res. 41:361, 1981; Herberman, R.B. Cancer Res. 19:207, 1974; Kedar, E., et al. Adv. Cancer Res. 38:171 1983; North, R.J. Adv. Immunol. 35:89, 1984 and Weiss, P.W., Curr. Topics Micro. Immunol. 89:1,1980). Mouse monoclonal antibodies also have not yet provided conclusive identification of a human tumor-specific antigen, although a few possibilities have been reported (Schlom, J., et al. (1985) Adv. Cancer Res., 43:143-174; Tsuji, Y., et al. (1985) Cancer Res., 45:2358-2362; Tong, A.W., et al. (1984) Cancer Res., 44:4987-4992; Chin, J., et al. (1985) Cancer Res. 45:1723-1929). However, monoclonal antibodies to many new differentiation antigens have been obtained, and some of these have been recognized as having potential value in tumor diagnosis and therapy, particularly if they are expressed at higher levels in tumors than in normal cells. It should be considered that even monoclonal

antibodies reacting with numerous normal cell types are more specific than current therapeutic agents (drugs and radiation). In addition several antigens identified by monoclonal antibodies (all of which are mucin-like) appear to be valuable serum markers for particular cancer types (Herlyn, M., et al. (1982) J. Clin. Immunol., 2:135-140; Klug, T.L., et al. (1984) Cancer Res., 44:1048-1053; Lan, M.W., et al. (1985) Cancer Res., 45:305-310; Papsidero, L.D., et al. (1984) Cancer Res., 44:4653-4647, Hirota, M., et al. (1985) Cancer Res., 45:1901-1905).

Ovarian carcinoma is a promising target for monoclonal antibody therapy (as all cancers of other non-essential organs) in that tissue-specific differentation antigens are as useful as tumor-specific antigens. However, monoclonal antibodies specific for a particular epithelial cell type have been difficult to obtain. Of the large number of monoclonal antibodies obtained reacting with human carcinomas, only a few appear to be specific for a particular histological type, namely the prostate (Raynor, R.H., et al. (1984) J. Nat. Cancer Inst., 73:617-625; Frankel, A.E., et al. (1982) Proc. Natl. Acad. Sci. USA, 79:903-907), the lung (Stahel, R.A., et al. (1985) Int. J. Cancer 35:11-17) and the breast (Menard, S., et al. (1983) Cancer Res., 43:1295-1300) and the breast, and these specificities have not yet been independently confirmed.

A number of ovarian tumor antigens have been detected using xenogeneic polyclonal sera (reviewed in Lloyd, K.O. (1982) Serono Symposium No. 46 (M.I. Colnagki, G.L. Buraggi and M. Ghrone, Eds.) Academic press. N.Y. pp. 205-211) but none are related to the antigens of the invention. Other laboratories have also described monoclonal antibodies to human ovarian carcinoma different from those of the invention. Bhattacharya et al. (Bhattacharya, M., et al. (1982) Cancer Res., 42:1650-1654) produced an antibody to a saline-extracted antigen detected only in mucinous cyst adenocarcinomas of the ovary and in fetal intestine. Serous cyst adenocarcinomas, the most common ovarian carcinoma, did not contain this antigen. Bast et al. produced an antibody (OC 125) reactive with an antigen present on 6/6 ovarian carcinoma cell lines and one melanoma of 14 non-ovarian cell lines tested. This antibody reacted with sections of 12/20 ovarian carcinomas and was nonreactive with 12 non-ovarian carcinomas and with most normal tissues, including normal adult and fetal ovary. Weak reactivity was observed with adult fallopian tube, endometrium and endocervix (Bast, R.C., et al. (1981) J. Clin. Invest., 68:1331-1336; Kabawat, S.E., et al. (1983) Amer. J. Clin. Pathol., 79:98-104).

We herewith incorporate by reference our previous work in the field namely U.S. patent application S.N. 562,465, and Intern J. of Gynocol. Pathol. 4:121 (1985) concerning mAbs MH94, MF116, MD144, MH55, MF61, MH94 and MH99; also U.S. patent application 764,862 and J. Histochem. and Cytochem (1985) 33:1095-1102 concerning mAbs MU78, MT334 and MQ49 and U.S. patent application S.N. 556,579 and Hybridoma 2:pp 253-264 (1983) concerning mAb MH99.

Summary

Monoclonal antibody for ovarian cancers described. The antigenic profile of each of these monoclonal antibodies is presented with both serological and tissue reactivity studies in cancer and normal cell lines and tissue sections. These monoclonal antibodies form a panel useful for the diagnosis and therapy of ovarian cancers.

Ovarian epithelial cells form a simple cuboidal epithelium, and have no known function that distinguishes them from other epithelial cells; it is possible that there is no marker unique to these cells.

Also provided by this invention is an $F(ab')_2$ fragment of the monoclonal antibody MX35.

This invention also provides a method of detecting human ovarian cancer in a subject which comprises obtaining a suitable sample from the subject, contacting the suitable sample with an amount of the aforementioned $F(ab')_2$ MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to form a complex with an antigen present on human ovarian cancer cells if present in the sample, and detecting any complexes so formed, thereby detecting human ovarian cancer in the subject.

This invention further provides a method of treating human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, conjugated to a therapeutic agent, such as a radioactive therapeutic agent, effective to treat human ovarian cancer.

This invention further provides a method of detecting human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to specifically form a complex with an antigen present on human ovarian cancer cells if present within the subject, and detecting the detectable marker labelling the antibody so complexed.

Detailed Description of the Invention

This invention provides an $F(ab')_2$ fragment of the monoclonal antibody MX35.

Also provided is the $F(ab')_2$ MX35 fragment labeled with a detectable marker. Detectable markers useful in the subject invention can readily be ascertained by those of ordinary skill in the art. Useful detectable marker include, but are not limited to, enzymes, for example alkaline phosphatase or horseradish peroxidase;

substrates for enzymes; compounds capable of fluorescing, for example fluorescein; and radioactive marker. Examples of radioactive markers useful for the subject invention include, but are not limited to, radioactive isotopes, for example radioactive iodine such as ^{131}I or ^{125}I, radioactive technetium, and radioactive indium, and compounds containing such radioactive isotopes. Other detectable markers may be found by those of ordinary skill, and such markers are useful for purposes of the subject invention.

This invention also provides the aforementioned $F(ab')_2$ MX35 fragment conjugated to a therapeutic agent. Therapeutic agents useful for the subject invention are those therapeutic agents which kill cancer cells, that is cells having a malignant phenotype. Therapeutic agents capable of killing cells having a malignant phenotype are well known to those of ordinary skill in the art, and any such therapeutic agent may be used in the subject invention. Useful therapeutic agents include drugs capable of killing malignant cells, for example doxorubicin; toxins; bacterial toxins, for example cholera toxin; and radioactive therapeutic agents, such as radioactive isotopes, for example ^{131}I, radioactive technetium, and radioactive indium, or compounds containing such radioactive isotopes.

This invention also provides a method of detecting human ovarian cancer in a subject which comprises obtaining a suitable sample from the subject, contacting the suitable sample with an amount of the aforementioned $F(ab')_2$ MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to form a complex with an antigen present on human ovarian cancer cells if present in the sample, and detecting any complexes so formed, thereby detecting human ovarian cancer in the subject.

Suitable samples for purposes of the subject invention include tissue samples, such as tissue biopsies. The tissue sample may be obtained from normal-appearing ovarian tissue. In one embodiment, the normal-appearing ovarian tissue sample is obtained from a

subject who may be predisposed to developing ovarian cancer, for example a subject whose family has a history of ovarian cancer. The tissue sample may, however, be obtained from abnormal-appearing tissue, for example from a growth or tumor. Such abnormal-appearing tissue may be abnormal-appearing ovarian tissue, but may also be abnormally-appearing tissue of any organ in the peritoneal cavity of a subject.

Another suitable sample is a fluid sample, such as, but not limited to blood, plasma, serum, mucus (for example cervical mucus), fluid obtained from the peritoneal cavity, including ascites fluid (i.e. fluid which has collected in the peritoneal cavity of a subject as a result of infection occurring, for example, from a growth or tumor).

Other suitable samples useful for the subject invention may be ascertained by those of ordinary skill in the art.

Complexes are detected by detecting the detectable marker labelling the antibody fragment, and the means of detection will depend on the particular detectable marker chosen for use in the subject invention. For example, if the detectable marker is an enzyme, antibody fragment-antigen complexes may be detected by contacting the sample with a substrate for the enzyme and monitoring production of the enzyme product. As another example, if the detectable marker is a radioactive marker, the antibody fragment-antigen complexes may be detected by X-ray, by counting radioactive emissions, or by scintillation counting. An appropriate detection means may be ascertained by those of ordinary skill in the art.

In one embodiment, detecting human ovarian cancer in the subject comprises detecting a micrometastatic tumor or micrometastatic tumors in the suitable sample obtained from the subject. A micrometastatic tumor is a small tumor which has metastasized from an ovarian cancer tumor. In one embodiment, a micrometastatic tumor is less than about 1.5 cm in diameter. In another embodiment

a micrometastatic tumor is less than about 1 cm in diameter. In a further, a micrometastatic tumor is less than about 5mm in diameter, and in another embodiment a micrometastatic tumor is less than about 1mm in diameter.

In another embodiment, detecting human ovarian cancer in the subject comprises detecting an epithelial ovarian carcinoma or epithelial ovarian carcinomas in the subject.

This invention further provides a method of treating human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, conjugated to a therapeutic agent, such as a radioactive therapeutic agent, effective to treat human ovarian cancer.

For purposes of the subject invention, "treating human ovarian cancer" means killing ovarian cancer cells. Note that the ovarian cancer cells are not limited in location in the ovaries, but may comprise metastases or may be shed ovarian cancer cells located in other parts of the subject's body, for example in the peritoneal cavity of the subject.

Suitable modes of administration of antibody fragments are known in the art, and such method may be used in the invented treatment method. Examples of suitable forms of administration include intravenous injection and intraperitoneal injection. Administration may comprise administering the subject antibody fragment into the subject's peritoneal cavity.

The effective amount may be determined by methods known in the art. Typically, the subject is given a small dose of the antibody fragment conjugated to the therapeutic agent, and the dosage is increased until the subject cannot tolerate adverse side effects caused by the antibody fragment-conjugated therapeutic agent.

In one embodiment of the aforementioned method of treatment, the

human ovarian cancer cells comprise a micrometastatic tumor or micrometastatic tumors. Micrometastatic tumors are described above.

This invention further provides a method of detecting human ovarian cancer in a subject which comprises administering to the subject an amount of the aforementioned MX35 fragment, labeled with a detectable marker, effective to and under conditions permitting the fragment to specifically form a complex with an antigen present on human ovarian cancer cells if present within the subject, and detecting the detectable marker labelling the antibody so complexed.

Appropriate methods of detection, as described above, depend on the chosen detectable marker. As described above, an appropriate method of detection can be determined by one of ordinary skill in the art.

In one embodiment of the above-described invented method of detecting human ovarian cancer in a subject, detecting human ovarian cancer in the subject comprises detecting a micrometastatic tumor or micrometastatic tumors in the subject. Micrometastatic tumors are as described above. In a further embodiment, the micrometastatic tumor or micrometastatic tumors are located in the subject's peritoneal cavity.

Suitable modes of administration include intravenous injection and intraperitoneal injection. Administration of the antibody fragment labelled with the detectable marker may comprise administering the fragment into the subject's peritoneal cavity.

This invention will be better understood from the Examples in the experiments which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of, and are not intended to, nor should they be construed to, limit the invention as described more fully

in the claims which follow thereafter.

We have investigated monoclonal antibodies to ovarian carcinomas for 5 years in an attempt to identify tumor-specific or tissue-specific markers (Mattes, M.J., et al. (1984) Proc. Nat'l. Acad. Sci. USA, 81:568-572; Cordon-Cardo, C., et al. (1985) Int. J. of Gynocol. Pathol., 4:121-130; Mattes, M.J., et al. (1985) J. Histochem. Cytochem., 33:1095-1102). Initially cell lines were used for immunization and screening. Since ovarian carcinoma cell lines are very rare, and are likely to be nonrepresentative of tumors occurring in vivo, we began another study in which we immunized mice with fresh tumor specimens, and used frozen tissue specimens as targets for screening. Here we describe 3 mAbs produced in this way, and a fourth, previously undescribed, produced earlier by immunization with an ovarian carcinoma cell line. These 4 monoclonal antibodies react with most of all fresh ovarian carcinomas and with a distinct range of normal epithelial cells. We describe their reactivity with fresh ascites carcinoma cells, and their lack of reactivity with normal mesothelium; these properties show a potential use in the effective intraperitoneal immunotherapy. We here describe the reactivity of these monoclonal antibodies on a large panel of normal and malignant cell lines and on frozen sections of normal human tissues, as well as some biochemical characteristics of the antigens recognized.

Target cells. The origin and culture of cell lines derived form human tumors, normal human fibroblasts and normal kidney epithelial cells have been described (Mattes, M.J., et al. Proc. Natl. Acad. Sci. USA (1984) Supra). A summary of the cell lines used is given in Table I. Normal tissues were obtained at surgery or autopsy. The ovarian cyst used for screening hybridoma supernants was a serious cystadenoma. Ovarian adenocarcinomas tested included serous (7 specimens), endometrioid (2) and mucinous (1), tumor cell liver was obtained from human cancer serology laboratory and further tissue specimens from Dr. C. Cordon-Cardo and Dr. Virginia K. Pierce at SKI. Tissues were covered with O.C.T. Compound (Miles laboratories, Elkhart, IN) and frozen in a slurry of 2-methyl-butane cooled in liquid nitrogen.

Production of mouse monoclonal antibodies. The monoclonal antibodies described were obtained from 3 fusions. For the generation of MT179, (Balb/c X C57BL/6)F1 mice were immunized the the ovarian carcinoma cell line SK-OV-4. Intraperitoneal injections of approximately 0.1 ml of packed cells were given twice at an interval of two weeks. The other monoclonal antibodies were obtained after immunizing mice with a mixture of 4 fresh ovarian carcinoma specimens, including 2 samples of ascites cells and 2 solid

tumors, using approximately 10^7 x cells of each specimen. The mixture was suspended in 1.0 ml and injected i.p. three times at 3 week intervals. To immunize with solid tumor specimens, fragments of frozen tumor were thawed, placed in approximately 2 volumes of Dulbecco's phosphate-buffered saline (DPBS[3], Gibco, Grand Island, NY), teased with scalpels and pressed through a fine still screen. This preparation was stored frozen for subsequent injections. Ascites cells were prepared as described in the accompanying paper; after thawing, they were washed once with DPBS. Three days after the last injection, the fusion of immune spleen cells with mouse myeloma MOPC-21 NS/1 cells was performed as described (Mattes, M.J., et al. (1983) Hybridoma 2:253-264). Initially cells were plated in 480 wells (Costar #3524, 24-well plates). Hybridoma cultures were subcloned at least twice by limiting dilution in 96-well plates on a feeder layer of normal mouse spleen cells. For mAb at MT179, culture supernatants were tested for antibody activity on a panel of cultured cells consisting of immunizing cell line and other types of human tumor cells. For the other monoclonal antibodies, supernatants were tested for reactivity on cryostate sections of a benign ovarian cyst. The specificity of epithelial-specific supernatants was tested further on various frozen sections, cell lines, and ABO blood

group-related antigen preparations, as described below. Since several months were required for this specificity analysis, cells from all original wells were frozen in 10% dimethyl sulfoxide, using 2 vials for each well, just after the original supernatants were collected. For cloning, cells were thawed, grown briefly in a well of a 24-well plate, re-tested for antibody activity and cloned using our usual procedure above. Cloned hybridoma cells were injected subcutaneously into nu/nu mice. Sera from mice with progressively growing tumors were collected and used for serological and biochemical characterization. Antibody subclass was determined by immunodiffusion in agar with anti-Ig heavy chain-specific reagents (Bionetics, Kensington, MD).

Serological procedures. Red cell rosetting methods for adherent cultured for 1-4 days and nonadherent target cells were carried out as described previously (Farr, A.G., et al. (1981) J. Immunol. Methods 47:129-144; Graham, R.C., et al. (1965) J. Histochem. Cytochem. 13:150-152). The immune Rosetting assay was done as described (Carey, T.E., et al. (1976) Proc. Natl. Acad. Sci. USA 73:3278. For adherent target cells, 200-500 trypsinized cells were plated in 0.01 ml in wells of Terasaki plates (Falcon Microtest plates 3034) and cultured for 1-4 days. Nonadherent target

cells were attached to the wells by pretreating the wells for 45 min. at room temperature with Conconavalin A (Con A, grade IV, Sigma Chemicals, St. Louis Mo) at 1.0 mg/ml in DPBS. After washing the plates twice and blotting, target cells in DPBS were added and incubated for 45 min at room temperature Mattes, M.J. et al. J. Immunol. Meth 61:145 (1983). To test for neuraminidase sensitivity, target cells were treated for 1 hr at 37 with Vibrio cholerae neuraminidase (Calbiochem-Behring, La Jolla, CA) diluted 1:10 in 0.05 M citrate buffer pH 5.5, 0.1 M NaCl, 0.01 M CaC12.

Cytoplasmic antigens were detected using an immunoperoxidase method as described (Farr, A.G., et al. (1981) J. Immunol. Meth. 47:129; Graham, R.C. Jr., et al. (1965) J. Histochem. Cytochem. 13:150-152) washed twice with PBS, then fixed with 2.0% buffered formaldehyde (Farr, A.G., et al. (1981) J. Immunol. Methods 47:129-144) for 30 min. All incubations were at room temperature. After 2 washes with PBS, they were incubated with 0.05% NP40 in PBS for 15 min. After 2 washes with PBS, 5% fetal bovine serum (FBS), monoclonal antibody was added, starting with a 1:500 dilution. After a 45 min incubation, plates were washed twice and peroxidase-conjugated rabbit anti-mouse Ig (DAKO P161, Accurate Chemicals, Westbury NY) was added (prepared immediately before use by mixing 1.0 ml 0.05 M acetate buffer pH 5.0, 0.05 ml 3-amino-9-ethyl carbazole at 4.0

mg/ml in N,N-dimethylformamide, and 0.005 ml 3.0% hydrogen peroxide (Graham, R.C. Jr., et al. (1965) J. Histochem. Cytochem. 13:150-152). After 15 min, the plates were washed twice with PBS, once with water, and examined.

Immunoperoxidase staining of tissue sections using the ACC method was also carried out as described previously (Mattes, M.J., et al. (1985) J. Histochem. Cytochem. 33:1095-1102).

Procedures for absorption of Ab activity, using cells scraped from culture flasks, have been described [Hirota, M., et al. (1985) Cancer Res. 45:1901-1905]. To test heat stability of antigens, cells were heated to 100°C for 5 min, then washed once before use in absorptions. Blood leukocytes and erythrocytes were tested by immunofluorescence as described (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA 81:568-572), using the monoclonal antibody at 1/50 and fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) at 1/40. Reactivity with blood group A, B, H, Lewis^a, Lewis^b, X and Y determinants (Lloyd, K.O., et al. (1968) Proc. Natl. Acad. Sci. USA 61:1470-1477) was determined by a solid phase enzyme-linked immunoassay as described (Lloyd, K.O., et al. (1983) Immunogenetics 17:537-541), except that the antigen preparations were dissolved in water.

Biochemical analysis. Each antibody was tested for its ability to precipitate an antigen from the spent medium and from detergent-solubilized cell extracts after labeling by 3 methods: metabolic incorporation of [^{3}H]glucosamine (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA, 81:568-572), metabolic incorporation of [^{35}S]methionine (Mattes, M.J., et al. (1984) Supra) or chloramine T ^{125}I labeling of solubilized cell membranes (Mattes, M.J., et al. (1983) Hybridoma 2:253-264). NP40 solubilization of labeled cells (Mattes, M.J., et al. (1983) Supra) and immunoprecipitation procedures (Mattes, M.J., et al. (1984) Supra) have been described previously. To test heat stability, radio labeled extracts were heated at 100 for 5 min; precipitated proteins were removed by centrifugation (7,000 rpm, 15 min), then standard immunoprecipitations were performed. Preparation of chloroform; methanol, 2:1 cell extracts and their use in inhibitions assays has been described (Mattes, M.J., et al. (1984) Supra).

The examples below are for illustrative of the invention without limiting it.

Example I

Of the 4 monoclonal antibodies described here, one, MT179, was produced by immunization and screening with an ovarian carcinoma cell line, SK-OV-4. The assay employed was immunoperoxidase staining of fixed and permeabilized cells, which was intended to detect primarily cytoplasmic antigens. Initial selection of hybridomas to be cloned was based on nonreactivity with a panel of melanomas and astrocytomas, so we expected that monoclonal antibodies restricted to epithelial differentiation antigens would be selected. Other monoclonal antibodies produced from the same fusion were previously described (Mattes, M.J., et al. (1985) J. Histochem. Cytochem., 33:1095-1102). Three other monoclonal antibodies were obtained by immunization and screening with fresh tissue specimens. Immunization was with a mixture of 4 fresh ovarian carcinomas, as described. The mAbs were screened on cryostat sections of a benign serous ovarian cyst. This cyst specimen was chosen because the lining epithelial cells appeared morphologically similar to the simple cuboidal epithelial cells of the normal ovary, but were preserved better than normal ovarian epithelial cells in frozen sections. Therefore, this screening was primarily designed to detect tissue-specific epithelial differentiation antigens. Initial selection of hybridomas

was on the basis of reactivity with epithelial cells and non-reactivity with connective tissue and blood vessels in the sections. Of 393 supernatants tested, 16 were epithelial-specific. A much larger number, roughly 1/3 of the total supernatants, showed reactivity with all cells in the section. Before subcloning, we performed additional tests of specificity, in an effort to select ovarian-specific monoclonal antibodies. The 16 supernatants were tested on sections of normal colon and the 5 supernatants, which were negative were retained, these supernatants were also negative on normal skin. Since many epithelial differentiation antigens have been identified as ABO blood group-related antigens, we tested these five supernatants on a panel of mucins containing the antigens A, B, O, Le^a, Le^b, X and Y. Two of the supernatants reacted with the A antigen. The remaining 3 supernatants, designated MW162, MW207 and MX35, were then tested against cryostat sections of 5 solid ovarian tumors; all 3 reacted with 5/5 specimens. They were then tested on a panel of 30 cell lines, including ovarian carcinomas, other carcinomas and other tumor types. This was done partially to confirm the specificity of the antibodies, but also to select positive cell lines which could then be used as targets for screening subclones. Target cells were tested by 2 assays: a rosetting assay to detect cell surface antigens and an

immunoperoxidase assay on fixed, permeabilized cells to detect primarily cytoplasmic antigens. All three supernatant antibodies reacted with some ovarian carcinoma cell lines and were negative on melanoma and astrocytoma cell lines; they also reacted with some non-ovarian carcinoma cell lines. Frozen cells from these three original wells were thawed, established in culture, tested for retention of reactivity, then cloned twice by limiting dilution before expansion.

The four monoclonal antibodies described were all non-reactive with blood leukocytes and erythrocytes by immunofluorescence. Also, they did not react with any of the ABO blood group-related antigen preparations tested.

MT179. Ab MT179 is an IgG1. Although it reacts strongly in immunoperoxidase assays, it has not precipitated a detectable component from ovarian carcinoma SK-OV-4 or colon carcinoma SW 480 cells labeled with [^{35}S]methionine, [^{3}H]glucosamine of ^{125}I. In absorption experiments, the antigen was destroyed by heating at 100° for 5 min, suggesting that it is a protein. MT179 was detected initially by immunoperoxidase staining of fixed, permeabilized tissue culture cells, which produced cytoplasmic staining, but MT179 was also detected on the cell surface of SK-OV-4 by a rosetting assay.

In frozen sections, MT179 was detected in a number of normal epithelial cells, namely in the colon, lung, skin, pancreas and breast (Table 1). Other epithelial cells and all non-epithelial cells were negative. MT179 was detected also in sections of 8/10 ovarian carcinomas. Expression in 147 tissue culture cells lines is summarized in Table 2. MT179 was detected by immunoperoxidase staining in 3/8 ovarian carcinomas (SK-OV-4, SW626, A7), 2/2 uterine carcinomas (SK-UT-2, ME-180), 9/11 colon carcinomas (SW480, SW620, SW1116, SW1222, SW1417, SK-CO-1, -13, CaCO-2, 4J), 5/9 bladder carcinomas (Scaber, RT4, 5637, JON, SW780), 3/4 pancreatic carcinomas (CAPAN-1, -2, A3), 8/11 lung carcinomas (SK-LC-2, -3, -4, -5, -7, -9, -11, -12) 2/5 breast carcinomas (MCF-7, SK-BR-7), 2/17 renal carcinomas (SK-RC-17, -35), 1/2 prostate carcinomas (DU145), 1/1 bile duct carcinomas (Charles) and 1/1 choriocarcinoma (SVCC), with a reciprocal titer ranging from 500-8,000. It was negative on 21 melanomas, 16/17 astrocytomas, 5 neuroblastomas and 26 hematopoietic tumors of various types. Regarding cultured normal cells, 3 fibroblast cultures were negative and 2/3 kidney epithelial cultures were positive.

<u>MW162</u>. MW162 is an IgM. It precipitated an antigen from [^{3}H]glucosamine labeled SK-OV-6 that migranted at the top of a 9% acrylamide gel (greater than 300,000

daltons). The antigen could still be precipitated after incubating the labeled extract at 100 for 5 min. These characteristics suggest that the antigen is a carbohydrate determinant on a mucin or proteoglycan. The antigen was not detectably precipitated from cell extracts labeled with [^{35}S]methionine or ^{125}I. Reactivity of MW162 with SK-OV-4 was not affected by pretreatment of the cells, after permeabilization, with neuraminidase. The antigen was not detected, by inhibition, in a glycolipid fractions of SK-OV-6 prepared by chloroform: methanol extraction.

In frozen sections, MW162 was detected in many epithelial cells, namely in the esophagus, stomach, bronchus, lung, kidney distal tubules, pancreas, thyroid, uterus and breast (Table 2. Other epithelial cells, such as in the colon and skin, were negative, as were all non-epithelial cells examined. The antigen was detected in frozen sections of 10/10 fresh ovarian carcinomas. Often staining in sections was concentrated at the luminal edge of cells.

Its distribution in 105 tissue culture cells is shown in Table 2. It was detected most readily in the cytoplasm by immunoperoxidase staining, so this assay was used for screening tissue culture cells; the antigen was

detected weakly and inconsistently on the cell surface by rosetting. It was detected in 5/8 ovarian carcinomas (SK-OV-3, -4, -6, Colo 316, A10), 2/9 colon carcinomas (SW1116, SW1222), 3/6 bladder carcinomas (JON, VM-CUB-1, -2), 2/3 pancreatic carcinomas (CAPAN-1, -2), 6/8 lung carcinomas (SK-LC-1, -3, -7, -8, -17, -21), 4/4 breast carcinomas (MCF-7, SK-BR-5, -7, CAMA), 2/11 renal carcinomas (SK-RC-7, -18) and 1/1 choriocarcinoma (SVCC), with a reciprocal titer ranging from 500-2,000. It was negative on 10 melanomas, 10 astrocytomas, 4 sarcomas and 20 hematopoietic tumors of various types. Regarding normal cultured cells, it was negative on 4 fibroblast cultures and positive on 3/3 kidney epithelial cultures.

MW207. MW207 is an IgG1. Of the antigens described here, it is the only one that was recognized initially as a cell surface antigen, so the rosetting assay, rather than the immunoperoxidase assay, was used to screen cell lines. It has not precipitated a detectable component from ovarian carcinoma SK-OV-6 or renal carcinoma SK-RC-18 labeled by any of the 3 isotopes described in Materials and Methods. In absorption experiments, it was destroyed by heating to 100 for 5 min, suggesting that the determinant recognized is a protein. [Three ml of packed cells absorbed all detectable Ab activity, while 10 times more heat-treated cells had no absorption activity.]

In frozen sections, MW207 was detected on certain epithelial cells only, namely in the bronchus, lung, kidney proximal tubules, pancreas thyroid, uterus and breast (Table 1. In the pancreas, only the cells lining ducts were stained (Fig. 2). MW 207 was also detected in sections of 10/10 ovarian carcinomas. On 103 cell lines (Table 2) MW207 was present on 5/8 ovarian carcinomas (SW626, A7, A10, SK-OV-3, -6), 5/9 colon carcinomas (SW620, SW837, SW116, SW1222, SK-CO-10), 1/6 bladder carcinomas (VM-CUB-1), 2/3 pancreatic carcinomas (CAPAN-2, ASPC-1), 6/8 lung carcinomas (SK-LC-1,-7,-8,-14,-21,LcLL), 3/4 breast carcinomas (MCF-7, SK-BR-5, -7), 10/11 renal carcinomas (SK-RC-1, -7, -10, -15, -18, -29, -33, -42, -45, Caki 1), 1/1 teratocarcinomas (Tera-1) and 1/1 choriocarcinoma (SVCC) with reciprocal titers ranging from 10^3 to 10^5. The most strongly reactive target cells included carcinomas of the ovary, colon, bladder, lung, breast and kidney. MW207 was negative on 10 melanomas and 18 hematopoietic tumors, but reacted weakly with 2/10 astrocytomas and 1/4 sarcomas. Hence this Ab appears to be not strictly restricted to epithelial cells, although in frozen sections only epithelial cells were detectably stained. Regarding normal cells, MW207 was negative on 4 fibroblast cultures and positive on 2/3 kidney epithelial cell cultures.

MX35. MX35 is an IgG1. Although it reacted strongly in immunoperoxidase assays, it has not precipitated a detectable component from ovarian carcinoma SK-OV-6 or renal carcinoma SK-RC-18 cells, labeled by any of the 3 isoptopes described above. Heat stability could not be determined by absorption experiments, since the AB activity, diluted to near the endpoint, was not absorbed by an equal volume of unheated packed A10 cells, due perhaps to a low level of antigen exposure in scraped cells. MX35 was initially recognized as a cytoplasmic antigen, but was also detected by rosetting on the cell surface of the ovarian carcinoma line A10.

In frozen sections, MX35 was detected in epithelial cells of the normal bronchus, lung, kidney collecting ducts, thyroid and uterus (Table 1). All other tissues examined were negative. MX35 was also detected in sections of 10/10 fresh ovarian carcinomas. Staining was often concentrated at the luminal edge of cells. In tissue culture cell lines, MX35 expression was rare (Table 2), being detected on only 3/8 ovarian carcinomas (A7, A10, SK-OV-6), 1/8 lung carcinomas (SK-LC-1) and 3/11 renal carcinomas (SK-RC-18), -33, -53), with reciprocal titer of 500-32,000. The most strongly positive cell lines were SK-LC-1, SK-RC-18 and SK-RC-53. Since, as noted above,

10/10 fresh ovarian carcinomas were positive, the data suggests that this antigen may be lost during adaptation of tumor cells to tissue culture. Regarding normal cells, MX35 was negative on 4 fibroblast cultures and positive on 1/3 kidney epithelial cell cultures. Considering the results with both normal tissues and cell lines, MX35 is the most restricted of the antigens described here.

We have described above 4 distinct epithelial differentiation antigens identified by monoclonal antibodies. From their distribution on normal tissues and cell lines, it is clear that the 4 antigens are different from each other. We are not aware of antigens described by other investigators that are likely to be identical to these. Three of the monoclonal antibodies did not precipitate a detectable radio-labeled component, so little is known about the biochemical nature of the antigens recognized. The reason for the lack of immmunoprecipitation is not known, but possibilities include the following: 1) The antigen recognized is a minor cell constituent with a slow turn-over, so is not labeled adequately. 2) The antigen lacks glucosamine, methionine and tyrosine. 3) The antigen is either not extracted or denatured by the detergents used to solubilize the cells. MT179 and MW207 were heat labile, suggesting that they are proteins.

MAbs to differentiation antigens have a number of possible applications in cancer diagnosis and therapy as well as in more basic studies for cell biology and differentiation. In the example, we describe results of immunofluorescent staining of fresh ovarian carcinoma ascites cells, using these 4 monoclonal antibodies, which suggest that they are potentially useful in intraperitoneal therapy of such tumors. In addition, these Abs would probably be helpful in identifying rare carcinoma cells in peritoneal washings or lymph nodes, as has been described with other mABs (Ghosh, A.K., et al. (1983) J. Clin. Pathol. 36:1150-1153; and Johnston, W.W., et al. (1985) Cancer Res., 45:1894-1900). Also, a significant number of intraperitoneal carcinomas derive from an unknown or uncertain primary. These monoclonal antibodies can be helpful in determining the origin of the primary, but such an application would require prior extensive tests with frozen sections of tumors of known histological types. MX35 seems most useful in this regard, since it is the most restricted of the antigens described here, being negative on carcinomas of the colon, bladder, pancreas and breast, but positive on a proportion of carcinomas of the ovary, lung and kidney. Sections of 10/10 ovarian carcinomas (the only type of carcinoma tested) were positive. Although many normal epithelial cells express these antigens, there may be

certain tissue types in which the antigens are markers of malignancy. For example, MW162 and MW207 were negative on the normal colon, but were positive on some colon carcinoma cell lines. Further studies on cryostat sections of carcinomas of various types are required to investigate this possibility. Also, considering that most cancer serum markers defined by monoclonal antibodies have been characterized as mucins (Herlyn, M., et al. (1982) J. Clin. Immunol. 2:135-140; Klung, T.L., et al. (1984) Cancer Res., 44:1048-1053; Len, M.S., et al. (1985) Cancer Res., 45:305-310; Papsidero, L.D., et al. (1984) Cancer Res., 44: 4653-4647; Hirota, M., et al. (1985) Cancer Res., 45:1901-1905), the mucin-like antigen identified by MW162 should be a potential serum marker.

We have not yet obtained monoclonal antibodies to tumor-specific or tissue-specific antigens, and this negative result warrants some discussion. Other laboratories have had similar results, although one report of ovarian carcinomas tumor-specific monoclonal antibodies has recently appeared (Tsuji, Y., et al. (1985) Cancer Res., 45:2358-2362). We have performed 22 fusions after immunizing mice with various ovarian carcinoma cell lines. From these fusions, monoclonal antibodies that are tumor-or tissue-specific would have been detected, if they were

present on the immunizing cell line. We conclude that more restricted antigens, possibly: 1) Do not exist; 2) Are not readily detected by current methods; or, 3) Are not present on cell lines. The few ovarian carcinoma cell lines are not likely to be representative of in vivo tumors; the difficulty in establishing new lines of ovarian and other carcinomas (except renal) is well known. In this paper we present our results of 2 initial fusions using fresh tissue as immunogen and screening target, and we believe further similar studies will be productive. The strategy of freezing uncloned hybridomas, to allow time for specificity testing on frozen sections, made this approach possible, and should be widely applicable.

Example II

Example for Therapy

We have attempted to select monoclonal antibodies that might also be effective agents for diagnosis and intraperitoneal therapy or radioimmunodetection of human ovarian carcinoma. Antibodies were tested for reactivity with the surface of fresh ovarian carcinoma ascites cells, and for non-reactivity with normal mesothelial cells. The antibodies tested included 33 that had been identified previously as reacting with epithelial differentiation antigens. Five antibodies were selected with the desired

specificity, MH99, MT179, MW162, MW207 and MX35, and these antibodies also reacted with cryostat sections of most of all ovarian carcinomas and benign ovarian cysts. All reacted also with certain normal epithelial cells. We also observed that the degree of heterogeneity of antigen expression on ascites carcinoma cells was dependent on the particular antigen being examined, and related to the biochemical nature of the antigen. In particular, most ABO blood group-related antigens showed a striking degree of heterogeneity. The rationale for intraperitoneal immunotherapy and the criteria for selecting appropriate antibodies are discussed.

The effectiveness of monoclonal antibodies in cancer immunotherapy and immunolocalization depends on their specificity. The optimal target antigen would be tumor-specific, and much effort has been directed to obtain such monoclonal antibodies. To date, no monoclonal antibody has been conclusively shown to identify a tumor-specific antigen; though there are some possible candidates for breast carcinoma (Schlom, J., et al. (1985) Adv. Cancer Res., 43:143-174), ovarian carcinoma (Tsuji, Y., et al. (1985) Cancer Res., 45:2358-2362), pancreatic carcinoma (Chin, J., et al. (1985) Cancer Res., 45:1723-1729) and lung

small cell carcinoma (Tong, A.W., et al. (1984) Cancer Res., 44:4987-4992), but characterization of these antigens is still preliminary. The search for tumor-specific antigens is based on numerous prior immunological studies suggesting the presence of such antigens on human tumors (Old, L.J., et al. (1981) Cancer Res., 41:361-375; Herberman, R.B., et al. (1974) Adv. Cancer Res., 19:207-263; Kedar, E., et al. (1983) Adv. Cancer Res., 38:171-288; Szigeti, R., et al. (1985) Adv. Cancer Res., 43:241-306; Shuster, J.,e t al. (1980) Prog. Exp. Tumor Res., 25:89-139; and Thomson, D.M.P., et al. (1985) Int. J. Cancer 35:707-14). The published evidence primarily consists of data indicating an immune response to syngeneic tumors, as detected by assays for antibodies (Old, L.J., et al. (1981) Supra), lymphocyte-mediated growth inhibition or cytotoxicity (Herberman, R.B., et al. (1974) Supra), T lyymphocyte-mediated cytotosicity (Kedar, E., et al. (1983) Supra), delayed hypersensitivity (Herberman, R.B., et al. (1974) Supra), macrophage migration inhibition (Szigeti, R., et al. (1985) Supra) or leukocyte adherence inhibition (Shuster, J., et al. (1980) Supra and Thomson, D.M.P., et al. (1985) Supra). However, such approaches have not yet allowed definite characterization of any tumor antigen, and the results have generally not been consistently reproduced in different laboratories, so the presence of human

tumor-specific antigens must still be considered speculative (discussed in references Schlom, J., et al. (1985) Supra; Weiss, D.W., et al. (1980) Curr. Topics Micro. Immunol. 89:1-83; North, R.J., et al. (1984) Adv. Immunol., 35:89-156).

Although tumor-specific monoclonal antibodies are not available, monoclonal antibodies to differentiation antigens may be of value. Such Abs react with certain normal adult cells as well as tumor cells of particular types, so toxicity arising from reactivity with normal cells is probable with some of all of such monoclonal antibodies. However, by adjusting the dose and by modifying the monoclonal antibody (such as by preparation of antibody fragments, or conjugates with radioisotopes or toxins) it may be possible to obtain a therapeutic effect without major side effects. It should be considered that such monoclonal antibodies are more specific than current therapeutic agents. Chemotherapeutic agents were selected initially not for their specificity, but for their toxicity, and effective treatment requires the maximum tolerated dose; the same approach seems valid for mAB therapy. The major difference in this regard between monoclonal antibodies and new chemotherapeutic drugs is that monoclonal antibodies cannot be pretested in animals; this makes determination of the optimal treatment regimen much more difficult.

We have focused on selecting mABs for therapy and radioimmunodiagnosis of ovarian carcinoma. This tumor type is the leading cause of death among patients in the United States with gynecologic malignancy, and is not treated effectively by current methods (Bender, H.G., and Beck, L. (Eds) (1983) Carcinoma of the Ovary. New York:Gustav Fischer Verlag, 1983 and Haije, W.G., et al. (1982) Ann. Clin. Biochem., 19:258-262). Radioimmunodetection might decrease the need for "second-look" surgery, which is currently performed routinely to diagnose tumor recurrence. In regard to monoclonal antibody therapy, ovarian carcinoma has two major advantages. First, as with other non-essential organs, a tissue-specific antigen would be as useful as a tumor-specific antigen. This advantage however has not yet materialized, in that no differentiation antigen specific for ovarian epithelial cells has yet been described. Second, the tumor grow primarily in the peritoneal cavity; blood-borne metastases can occur, but most patients succumb prior to this (Bergman, F., et al. (1966) Acta. Obstet. Gynecol. Scand., 45:211-225). Therefore, intraperitoneal therapy wold be expected to enhance interaction of the monoclonal antibody with tumor cells and to reduce interaction with normal, antigen-positive cells outside the peritoneal cavity. The importance of this factor is difficult to evaluate at

present. Serum proteins pass rapidly from the peritoneal cavity to the blood in normal animals (French, J.E., et al. (1960) Quart. J. Exper. Physiol., 45:88-103).

Antibodies injected i.p. into patients with ovarian carcinoma are initially exposed to only one type of normal cell, mesothelial cells, which line all surfaces of the peritoneal cavity. In this paper we describe the selection of monoclonal antibodies that react with the surface of fresh tumor cells but not with normal mesothelium. Fresh ascites carinoma cells were used as targets in immunofluorescence. Ovarian carcinomas grow as both ascites and solid modules of tumor cells attached to the lining of the peritoneal cavity. To predict reactivity of monoclonal antibodies with tumor cells in vivo, use of ascites cells as targets seems more reliable than examining either tissue culture lines of ovarian carcinoma (which are rare and probably not representative) or frozen sections of fresh tumors (which would not indicate which antigens are accessible on the cell surface in vivo). Mesothelial cells were examined in frozen sections. Also examined were frozen sections of solid ovarian carcinomas and benign ovarian cysts. The monoclonal antibodies tested were 33 Abs produced by our laboratories which, from previous studies, appeared to demonstrate specificity for epithelial cells,

and included monoclonal antibodies reacting with ABO blood group-related antigens. We have identified 5 distinct monoclonal antibodies that reacted with the surface of most or all fresh tumor specimens and that were negative on mesothelium; they also reacted with various normal epithelial cells. These antibodies appear suitable for further evaluation as potential therapeutic agents.

Antibodies. The 33 monoclonal antibodies tested in this study were generated and characterized previously. They were obtained from mice immunized with various human and were included in these experiments on the basis of preferential reactivity with epithelial cells in frozen sections of normal human tissues and in cultured cell lines. All have been tested on a wide range of cell lines and normal tissues, and also have been partially characterized biochemically by immunoprecipitation (which in some cases did not precipitate a detectable component). Based on these data, the monoclonal antibodies appeared different from each other. In the following list, they are grouped according to the type of carcinoma used for immunization and to the publication in which they are described: ovarian, MF61, MF116, MH55, MH94 (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA 81:568-572; Cordon-Cardo, C., et al. (1985) Int. J. Gynecol. Pathol., 4:121-130), MH99 (Mattes, M.J., et

al. (1983) Hybridoma 2:253-264), MQ49, MT334 (Mattes, M.J., et al. (1985) J. Histochem. Cytochem., 33:1095-1102), MT179, MW162, MW207, MX35 (Mattes, M.J., et al. Four mouse monoclonal antibodies to human epithelial differentiation antigens above), MR54, MT78, MV9 (M.J. Mattes, unpublished data); bladder, T16, T87 (Fradet, Y., et al. (1984) Proc. Nat. Acad. Sci. (Wash.), 81:224-228); renal, S6 (Ueda, R., et al. (1981) Proc. Nat. Acad. Sci. (USA), 78:5122-5126); teratocarcinoma, K4 (Rettig, W.J., et al. (1985) in press); choriocarcinoma, LK26, SV19, SV63 (placental alkaline phosphatase) (Rettig, W., et al. (1985) Int. J. Cancer 35:469-475); lung, F-15, F-16 (J. Feikert, unpublished data and U.S. patent application S.N. 474,225); and colon, HT29-15, V-215, CLK314 (Sakamoto, J., et al. (1985) Fed. Proc. 42:792). We also tested 7 monoclonal antibodies to ABO blood group-related antigens, since these are epithelial differentiation antigens. These monoclonal antibodies also were obtained after immunizing mice with various human carcinomas, and include: anti-A, HT29-36 (Furukawa, K., et al. J. Immunol., in press); anti-B (Ueda, R., et al. (1981) Supra) anti-Lewisa, T174; anti-Lewisb, T218; anti-H type 2, H11; anti-X, P12; and anti-Y, F3 (Sakamoto, J., et al. (1984) Molecular Immunol., 21:1093-1098).

Ascites cells. 0.5-2.0 l ascites fluid from patients with serous adenocarcinoma of the ovary were

filtered through 4-ply gauze and spun 5 min at 600g. Pelleted cells were resuspended in 5-10 volumes of supernatant, and 40 ml aliquots were underlaid with 10 ml Ficoll-Paque (Pharmacia, Piscataway, NJ). After spinning 15 min at 3,000g, the cells at the interface were collected, washed once with Dulbecco's phosphate-buffered saline (DPBS, Gibco, Grand Island, N.Y.), 7.5% fetal calf serum, 10% dimethylsulfoxide at various concentrations (1-10% packed cell volume/volume) and frozen in liquid nitrogen. Immunofluorescent staining was performed by standard procedures (Mattes, M.J., et al. (1984) Proc. Natl. Acad. Sci. USA 81:568-572), using mAb sera at 1/50, fluorescein-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) at 1/40 in medium containing 20% normal human serum, and 3-6 ul packed ascites cells per sample. Examination was by epi-illumination using a 75W xenon lamp, Leitz filter cube H, and a 40x objective. Some samples contained normal cells such as lymphocytes, macrophages and mesothelial cells as well as tumor cells; therefore, evaluation was based on observation of clustered cells only, since carcinoma cells in ascites usually are in clusters, while the normal cells present are rarely in clusters. Samples of cells were processed and examined by the Pathology Laboratory at SKI (Sloan-Kettering Institute, N.Y., N.Y.) (Dr. Patricia Saigo), to confirm that virtually

all cells in clusters were malignant. Photographs of immunofluorescence were prepared using Kodak Tri-X film and 90 second exposures. Bone marrow cells from normal donors were provided by the Bone Marrow Transplantation unit at SKI and examined similarly by immunofluorescence.

Cryostat sections. General methods for staining 0.007 mm sections by the avidin-biotin complex method have been described (Mattes, M.J., et al. (1985) J. Histochem. Cytochem. 33:1095-1102). Ovarian cysts included 1 serous cystadenoma, 3 mucinous cystadenomas, 1 simple cyst, 1 serous cystadenocarcinoma of low malignant potential, and 1 mucinous cystadenocarcinoma of low malignant potential. Strips of the cyst wall were folded in pleats before freezing. Tissues containing normal mesothelium included the diaphragm, body wall and pericardium. Ovarian carcinomas included serous (7 specimens), mucinous (1 specimen) and endometroid (2 specimens).

The 33 monoclonal antibodies evaluated were generated in our laboratories by immunization with ovarian, bladder, renal, lung, and colon carcinomas and with choriocarcinomas. We anticipated that the expression of cell surface antigens on ovarian carcinoma ascites cells might be quite different from antigen expression on cultured

cell lines, and therefore we tested 26 monoclonal antibodies reacting with a variety of epithelial differentiation antigens. ABO blood group-related antigens are epithelial differentiation antigens; therefore, a panel of 7 monoclonal antibodies to blood group-related structures, which were also obtained after immunizing mice with human carcinomas, was included.

As the initial screening for a most of the monoclonal antibodies, we examined frozen sections of a benign ovarian cyst, to confirm the specificity of the monoclonal antibodies for epithelial cells. A benign cyst was used rather than a normal ovary because, in our experience, epithelial cells are better preserved in frozen sections. Monoclonal antibodies reacting with connective tissue or blood vessels were eliminated at this stage, which included 8 monoclonal antibodies (MR54, T16, T87, V-215, CLK314, F-15, F-16 and LK29). None monoclonal antibodies produced the expected staining of epithelial cells only. Eight monoclonal antibodies were negative on the benign cyst, but were tested further for reactivity with fresh ascites cells. We observed that some monoclonal antibodies stained the outer surface of the cyst as well as the inner epithelial cells. The cells lining the outer surface are presumably drived from the normal ovarian epithelium, but

due to cyst formation they appear flattened, like mesothelial cells, in morphology. The monoclonal antibodies that stained the outer as well as the inner surface of the cyst, (MH94, MQ49, MT334, HT29-15 and F3), all react with heat stable (100°) antigens, which are probably carbohydrate. One, F3, reacts with the Y blood group-related antigen. Another, MQ49, reacts with both glycolipids and mucin-like molecules (Mattes, M.J., et al. (1985) Supra). MH94, MT334 and HT29-15 also react with mucin-like molecules. The MH94 antigen was detected by immunoprecipitation after labeling with $H_2{}^{35}SO_4$, but not with [3H]glucosamine (Mattes, M.J., et al. Proc. Nat'l. Acad. Sci USA 81:568 (1984) and unpublished data). Other monoclonal antibodies (MH99, MT179, K4 and SV19) reacted with cells lining the inner surface of the cyst only.

The 25 remaining monoclonal antibodies were tested against at least 2 specimens of fresh ovarian carcinoma ascites cells, by immunofluorescence. We had initially attempted, to facilitate assays, to attach these target cells to wells of Terasaki plates using concanavalin A, which has been effective with a wide range of nonadherent cell types (Mattes, M.J. et al., J. Immunol. Meth. 61:145 (1983). However, the ascites cells did not attach stably under these conditions, suggesting that they have unusual

surface properties. We also tested 2 broadly reactive monoclonal antibodies, MA103 and AJ2 (Mattes, M.J., et al. (1983) Hybridoma 2:253), which are present on all human tissue culture cells: these monoclonal antibodies reacted with all ascites cells tested. Monoclonal antibodies negative with the 2 ascites specimens were not tested further (11 monoclonal antibodies: MF61, MF116, MH55, MV9, K4, SV19, SV63, S6, HT29-15, HT29-36 and S8), but positive monoclonal antibodies were tested on additional ascites specimens. Results are summarized in Table 3. Six monoclonal antibodies reacted with 5/5 or 4/5 specimens, and produced fairly homogeneous, ringed staining of tumor cells 2E). In these studies, we examined primarily clumped tumor cells since these were identified as malignant. The nature of the single cells was more variable, but many specimens clearly contained many malignant single cells, as indicated both by morphology and immunofluorescent staining.

Several monoclonal antibodies produced staining with certain unusual characteristics: 1) Tumor cells had a striking degree of heterogeneity. The fraction of positive tumor cells (in clumps) ranged from a few per cent to 50%. Positive cells were often extremely bright, although brightness was variable, so the positive and negative cell populations appeared to be distinct. A single clump of

cells usually contained a mixture of strongly positive and negative cells. 2) In some specimens, many positive cells were not ringed, but were stained over a continuous portion, generally 1/3 to 2/3, of their surface. This occurred in both clumped cells and single tumor cells. In clumped cells, the stained portion of the membrane was usually the area not in contact with another cell. This antigen distribution did not appear to be antibody-mediated, since it occurred in cells stained at 4° in the presence of 10 mM NaN3 (and which had been pretreated for 30 min with NaN3 before the first antibody incubation).

Of the monoclonal antibodies to ABO blood group-related antigens tested, most that were positive on asciates cells produced this heterogeneous pattern, and this occurred with most specimens examined. The only other monoclonal antibodies producing this heterogeneous pattern were MQ49 and HT29-15, which also recognize carbohydrate determinants (Mattes, M.J., et al. (1985) J. Histochem. Cytochem. 33:1095 Supra and Sakamoto, J., et al. (1985) Fed. Proc., 42:792). The exception among blood group-related monoclonal antibodies was F3 (anti-Y) which produced bright, ringed staining of most or all tumor cells in all specimens examined. This antigen, however, is present on erythrocytes (strongly on type O, weakly on types A & B) (Sakamoto, J.,

et al. (1984) Molecular Immunol., 21:1093-1098). Therefore, it does not appear to be a suitable target for tumor localization or therapy and has not been included in our subsequent studies of monoclonal antibody specificity.

The 5 monoclonal antibodies consistently reactive with ascites cells were tested further for specificity and results are included in Table 3. They reacted with frozen sections of most of all ovarian carcinomas tested, and with the epithelial cells of many benign ovarian cysts. The most consistently positive monoclonal antibody was MH99, which reacted without exception with all cells derived form the ovarian epithelium. Table 4 summarizes the normal tissue reactivity of these monoclonals, which was described above. MH99 is most consistently positive and reacts with nearly all normal epithelial cells. THe other mAbs react with a subset of normal epithelial cells. Normal mesothelial cells in frozen sections of the lower surface of the diaphragm, body wall and pericardium were negative with all 5 mAbs. As described (Mattes, M.J., et al. (1983) Hybridoma 2:253 and "four mouse monoclonal antibodies to human epithelial differentiation antigens" Proc. Natl. Acad. Sci. USA) blood leukocytes and erythrocytes were negative by immunofluorescence. Normal bone marrow cells, examined by immunofluorescence, were also negative.

The pattern of staining in frozen sections varied depending on the particular antibody. Antibodies MH99, MT179, and MW207 stained all sides of the cells equally, in a pattern suggestive of membrane staining. In contrast, antibodies MW162 and MX35 often stained only the luminal edge of the cells and sometimes also produced a granular staining pattern.

To determine whether antibody-induced capping or other type of modulation of surface antigens occurs, immunofluorescent staining of 2 ascites specimens was performed in the absence of NaN3. Following staining, cells were incubated for 45 min at 37. This treatment had no effect on antigen distribution, relative to a control stained in the presence of NaN3, with any of the 5 monoclonal antibodies tested. We conclude that capping does not readily occur.

We also investigated the possibility that large amounts of soluble antigen in ascites fluid might inhibit monoclonal antibody binding in vivo. Fifty ul of autologous ascites fluid, collected at the same time as the cell specimen, was included during the first antibody incubation. This produced no detectable inhibition of immunofluorescent staining with any of the 5 monoclonal antibodies tested,

suggesting that such inhibition would not occur in vivo.

We have identified 5 monoclonal antibodies that have potential value in intraperitoneal immunotherapy or immunodiagnosis of ovarian carcinoma, since they react with the surface of fresh tumor cells but are negative on normal mesothelial cells. We attempted to examine cells as similar as possible to the cells that would be encountered by a monoclonal antibody injected i.p. We plan to examine cells from biopsies obtained following injection of radio-labeled antibody, to confirm the reactivity of the monoclonal antibodies in vivo. It is evident that examination of ovarian carcinoma cell lines, or cryostat sections of fresh tumors, does not provide reliable information regarding antigen expression on the surface of cells in vivo. For example, MT334 reacted with frozen sections of 10/10 tumors, but reacted weakly with only 1/5 ascites specimens.

The monoclonal antibodies described all react with some normal adult cells, but they may be effective in tumor localization or therapy without producing unacceptable side-effects. It must be considered that all current forms of cancer therapy are toxic, and the antibodies described are more specific than effects of chemotherapy or radiation. Moreover, after i.p. injection it is uncertain how

efficiently the antibodies will reach the positive normal cells. For example, antigens located at the luminal edge of epithelial cells, such as MW162 and MX35, may not be exposed to the circulating antibodies. Also IgM's such as MW162 may not penetrate tissues sufficiently to reach the positive normal cells. If the antibody is fragmented or conjugated to other molecules, this may strongly affect the localization in normal tissues and the side effects. In addition, monoclonal antibody binding to normal tissues may or may not produce toxicity. Radioisotope-conjugated monoclonal antibodies may damage tumor cells, which are relatively radio-sensitive, much more readily than normal cells to which they bind. Experimentation in humans is required to resolve these questions. Initially the localization of radiolabeled antibodies will be investigated to determine whether the monoclonal antibodies bind effectively to tumor and/or normal tissues. These experiments will also indicate whether the monoclonal antibodies might be useful for radioimmunodetection of small tumor masses, which might eliminate in some cases the need for "second-look" surgery to detect tumor recurrence.

The answer to this question is obcured by a large number of reports describing putative tumor-specific antigens (Old, L.J., (1981) Supra; Herberman, R.B. (1974) Supra; Kedar, E., et al. (1983) Supra; Szigeti, R. (1985) Supra; Shuster, J., et al. (1980) Supra; Thomson, D.M.P., et al. (1985) Supra; Weiss, D.W., (1980) Supra and North, R.J. (1984) Supra). Until such antigens have been well-characterized, which has not been achieved in a single case, they must be considered as speculative. A more tissue-specific monoclonal antibody, reactive with fewer normal cells, is clearly preferable, but it should be considered that ovarian epithelial cells are relatively undifferentiated, and produce no known specific differentiation marker. Moreover, the truly tissue-specific markers, such as proteins secreted by the prostate, pancreas or breast, are often lost as a tumor progresses to a less differentiated state. We emphasize that the choice of the optimal target antigen for therapy depends not only on its restricted distribution. Equally important is its consistent expression on nearly all tumor cells, at least from a particular patient, as well as on a low frequency of antigen-negative variants.

The monoclonal antibodies used in these studies were generated in our laboratories, and may or may not be the optimal monoclonal antibodies for i.p. immunotherapy.

Other laboratories also have attempted to generate monoclonal antibodies specific for ovarian carcinoma. Bast et. al. (Kabawat, S.E., et al. (1983) Int. J. Gynecol. Pathol., 2:275-285) described monoclonal antibody OC125, which recognizes a high molecular weight antigen that appears to be a valuable serum marker for ovarian carcinoma (Klung, T.L., et al. (1984) Cancer Res., 44:1048-1053). In cryostat sections of normal tissues, only the epithelia of the uterus, fallopian tube and endocervix, and mesothelial cells of the peritoneum, pleura and pericardium were positive. Approximately 85% of ovarian serous carcinomas were positive. The reactivity with normal mesothelial cells causes this monoclonal antibody to be apparently, inappropriate for i.p. immunotherapy. Also, expression of OC125 appeared to be heterogeneous in cryostat sections, with considerable negative cells being detected in positive tumors (Kabawat, S.E., et al. (1983) Amer. J. Clin. Pathol. 79:98-104). Gangopadhyay et al. (Gangopadhyay, A., et al. (1985) Cancer Res., 45:1744-1752) described the 1D3 monoclonal antibody, which reacted with essentially all ovarian mucinous carcinomas, and with only the normal colon among normal tissues examined; however, the more common serous carcinomas were negative. Tagliabue et al. (Tagliabue, E., et al. (1985) Cancer Res., 45:379-385) obtained 2 monoclonal antibodies to ovarian carcinoma, MOv1

and MOv2. Mov2 reacted with most but not all ovarian carcinomas of all types, and with normal colon, stomach and breast. It reacted by immunofluorescence with most but not all fresh ovarian carcinoma ascites cells. MOv1 reacted with mucinous but not serous ovarian carcinomas, and also reacted with the normal colon. Tsuji et al. (Tsuji, Y., et al. (1985) Supra) recently described 2 monoclonal antibodies: 4C7 reacted with most ovarian mucinous, endometroid and mesonephroid, but not serous carcinomas, while 3C2 reacted with most serous and endometroid, but not mucinous or mesonephroid carcinomas. These 2 antibodies did not react with any normal tissues, benign ovarian tumors or carcinomas of other organs.

Three monoclonal antibodies obtained to breast-carcinoma-associated antigens have been found to react with ovarian carcinoma ascites cells. F36/22 reacted with 47/47 ascites specimens, and was negative with normal mesothelial cells in the same specimens (Croghan, G.A., et al. (1984) Cancer Res., 44:1954-1962). This monoclonal antibody also reacted with sections of ovarian carcinomas, but not with the normal ovarian epithelium, while benign ovarian tumors had weak and variable staining. However, many normal cells were positive with F36/22, including the breast, lung, sebaceous gland, sweat gland, uterus and

kidney. Monoclonal antibody 3.14.A3, later called HMFG2, reacted with ovarian carcinomas and with normal bile duct, pancreas, sebaceous bland, salivary gland, kidney, lung, sweat gland and uterus (Arklie, J., et al. (1981) Int. J. Cancer, 28:23-29). This antibody, 131-I-labeled, was used in preliminary studies of i.p. injection for therapy of ovarian carcinomas (Epenetos, A.A., (1984) The Lancet, 1441-1443).

B72.3 has also been detected in fresh ovarian carcinoma ascites cells (Johnston, W.U.V., et al. Cancer Res. 45:1894 (1985). It is positive on breast and other carcinomas, but negative on all normal tissues examined. In sections of breast carcinomas, there was marked heterogeneity in the expression of B72.3 (Schlom, J. et al. (1985) Supra). The presence of other well-characterized tumor markers, including carcinoembryonic antigen, alpha-fetoprotein and human chorionic gonadotropin, was also investigated in ovarian carcinomas; all were negative on more than 75% of serous carcinoma (Casper, S., et al. Am. J. Obstet. Gynecol. 149:154 (1984).

An interesting observation was that many monoclonal antibodies to carbohydrate antigens produced striking heterogeneity in their staining of ascites cells. Tumor heterogeneity is of course a major obstacle in effective treatment, and our observations suggest the important point that the level of heterogeneity can vary widely depending on the particular antigen, and that this level may be related consistently with the biochemical nature of the antigen. The mechanism for variation in blood group antigen expression is unknown, but may be related to the altered and variable state of differentiation of tumor cells. We suggest that this heterogeneity is directly related to the fact that blood group carbohydrate antigens, including ABO, Ii and T, account for many of the most consistent differences between malignant and normal cells of the same histological type (Feizi, T., (1985) Nature 314:53-57). That is, expression of carbohydrate antigens may be a very sensitive indicator of the state of differentiation of a cell, allowing such antigens to be useful in diagnosis of malignancy. But, for the same reason, malignant cells may generally vary in expression of these antigens, making them unsuitable as targets for immunotherapy.

The rationale for i.p. immunotherapy is complex. Intraperitoneal therapy of ovarian carcinoma has been used extensively with a colloidal suspension of Cr32P04 (Rosenshein, N.B., et al. (1979) Obstet. Gyneco. Survey, 34:708-720) or with other chemotherapeutic drugs (Markman, M. in S.B. Howell (ed.) Intra-Arterial and Intracavitary Cancer Chemotherapy, PP. 61-69 Boston: Martiners Nijhoff, 1984), though evidently often without an understanding of the physiology of efflux from the peritoneal cavity, as noted by Leichner et al. (Leichner, P.K., et al. (1980) Radiology 134:729-734). Efflux of substances from the peritoneal cavity, including proteins, colloids, and erythrocytes, is very rapid via lymphatics of the lower surface of the diaphragm. The lack of a substantial barrier to efflux is due to the presence of specilized mesothelial cells and lymphatics at this location (French, J.E., et al. (1960) Quart. J. Exper. Physiol., 45:88-103). This factor reduces the advantage of intraperitoneal therapy. However, there are several factors that can enhance the effectiveness of this approach. First, as noted for drug therapy (Markman, M., (1984) In: S.B. Howell (ed.), Intra-Arterial and Intracavitary Cancer Chemotherapy, pp. 61-69. Boston: Martinus Nijhoff), the relative concentration in peritoneal fluid and in blood depends on both the rate of efflux from the peritoneal cavity and the rate of clearance from the

blood. Substances cleared rapidly from the blood are therefore preferable. The clearance rate of monoclonal antibodies from the blood might be increased in various ways, such as removing sialic acid (Ashwell, G., et al. (1974) Adv. Enzymol., 41:99-128). Second, patients with ovarian cancer have impaired efflux from the peritoneal cavity, due probably to blockage of lymphatics by tumor cells, which is presumably the reason for development of ascites (Feldman, G.B., et al. (1972) Cancer Res., 32:1663-1666 and Coates, G., et al. (1973) Radiology, 107:577-583). Third, it might be possible to decrease the rate of efflux in various ways which have been effective in experimental animals, such as by anaesthesia, blocking major lymphatic vessels (Courtice, F.C., et al. (1951) Austral. J. Exper. Biol. Med. Sci. 29:451-458), or disrupting the lymphatic capillaries on the lower diaphragm (Raybuck, H.E., et al. (1950) Am. J. Physiol. 199:1021-1024). Fourth, since the toxicity of monoclonal antibodies will result from binding to normal epithelial cells, if the antibody can be confined to the vascular system after it enters the blood, toxicity may be eliminated. This might be achieved by conjugating IgG to particles of diameter greater than 0.1u, which are too large to pass through the capillary endothelium (Renkin, E.M., et al. (1977) Circ. Res., 41:735-743 and Simionescu, N., et al. (1972) J. Cell Biol.

53:365-392). Regardless of the method of administration, some of the monoclonal antibody will enter the blood; this may be useful in that potentially metastatic tumor cells in the blood will be eliminated. The method developed for i.p. chemotherapy is to inject a large volume, and to withdraw it after several hours (Markman, M., (1984) Supra); this seems equally appropriate for monoclonal antibody therapy. Intraperitoneal immunotherapy for ovarian cancer has previously been attempted with polyclonal (Order, S.E., et. al. (1981) Cancer 48:590-596) and monoclonal (Epenetos, A.A., (1984) The Lancet, 1441-1443) antibodies in preliminary experiments. Clearly many variables must be investigated in order to devise the optimal approach.

These hybridoma cell lines are on deposit at Sloan-Kettering Institute 1275 York Avenue, New York, NY and at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, a recognized international depository, under the following designations:

SKI Number	ATCC Designation	ATCC Deposit Date
MF116	HB 8411	October 28, 1983
MD144	HB 8409	October 28, 1983
MH55	HB 8412	October 28, 1983

SKI Number	ATCC Designation	ATCC Deposit Date
MF61	HB 8410	October 28, 1983
MH94	HB 8413	October 28, 1983
MH99	HB 8406	October 28, 1983
ME195	HB 8431	November 16, 1983
ME46	HB8430	November 16, 1983
MW162		
MT179		
MW207		
MT35		
MU78	HB8877	July 15, 1985
MT334	HB8876	July 15, 1985
MQ49	HB8874	July 15, 1985

Table 1

Reactivity of moAbs with cryostat section of normal human tissues[a]

Tissue	MT179	MW162	MW207	MX35
Esophagus	-	+	-	-
Stomach	-	+	-	-
Colon	+	-	-	-
Epiglottis	nd[b]	-	-	-
Bronchus	nd	+	+	+
Lung	+	+	+	+
Skin	+	-	-	-
Kidney	-	+[c]	+[c]	+[c]
Pancreas	+	+	+	-
Liver	-	-	-	-
Thyroid	-	+	+	+
Ovary	-	-	-	-
Uterus	-	+	+	+
Breast	+	+	+	-
Testes	-	-	-	-
Heart	nd	-	-	-
Spleen	-	-	-	-
Lymph node	-	-	-	-
Thymus	-	-	-	-
Brain	-	nd	nd	nd

[a]The positive cells with these Abs were exclusively epithelial cells.

[b]Not determined

[c]In the kidney, MW207 reacted with proximal tubules, MW162 with Henle's loop and distal tubules, and MX35 with collecting tubules.

Table 2

Reactivity[a] of moAbs with tissue culture cell lines

Cell Type	MT179	MW162	MW207	MX35
Carcinomas				
Ovarian	3/8[b]	5/8	5/8	3/8
Uterine	2/2	0/2	0/2	0/2
Colon	9/11	2/9	5/9	0/9
Bladder	5/9	3/6	1/6	0/6
Pancreas	3/4	2/3	2/3	0/3
Lung	8/11	6/8	6/8	1/8
Breast	2/5	4/4	3/4	0/4
Renal	2/17	2/11	10/11	3/11
Prostate	1/2	0/1	0/1	0/1
Bile duct	1/1	-	-	-
Melanomas	0/21	0/10	0/10	0/10
Astrocytomas	1/17	0/10	2/10	0/10
Sarcomas	-	0/4	1/4	0/4
Teratocarcinomas	-	0/1	1/1	0/1
Choriocarcinomas	1/1	1/1	1/1	0/1
Neuroblastomas	0/5			
Hematopoietic tumors		-	-	-
T lymphocyte	0/8	0/7	0/7	0/7
B lymphocyte	0/9	0/4	0/3	0/4
Null lymphocyte	0/6	0/4	0/4	0/4
Myeloid	0/3	0/2	0/2	0/2
Monocytoid	-	0/2	0/1	0/2
Myeloma	-	0/1	0/1	0/1
Normal cells				
Fibroblasts	0/3	0/4	0/4	0/4
Kidneyepithelial	2/3	3/3	2/3	1/3

[a]MW207 was tested in a rosetting assay for cell surface antigens using a starting dilution of 1/1000. MT179, MW162 and MX35 were tested in a peroxidase assay on fixed, permeabilized cells, using a starting dilution of 1/500.

[b]Number positive/number different cell lines tested. The individual positive cell lines and the titers are listed in the text.

Table 3

Reactivity of moAbs with ovarian epithelial tumors

MoAb	Determinant	Ascites Cells: Positive	Ascites Cells: Heterogeneous	Frozen Sections: Benign Cyst Epithelia[d]	Frozen Sections: Carcinomas[d]
MH99	P[a]	5/5[b]	no	7/7	10/10
MI75	P	2/5	yes,10-50%	---	-----
MI179	?	4/5	no	5/5	8/10
MW207	?	5/5	no	3/6	10/10
MX35	?	5/5	no	3/6	10/10
MH94	C	1/5	yes,20%	4/6	4/9
MQ49	C	3/5	yes,10-50%	1/1	6/8
MI334	C	1/5	yes,30%	4/5	10/10
MW162	C	5/5	no	3/6	10/10
HT29-15	C	1/2	yes,5%	1/1	-----
H-11	C,H type 2	1/2	yes,2%	---	-----
T164	C,Le[a]	1/2	yes,2%	---	-----
T218	C,Le[b]	1/2	yes,1%	---	-----
F3	C,Y	5/5	no	1/1	-----
P12	C,X	3/5	yes,2-50%	0/1	-----

[a]P, protein; C, carbohydrate; ?, unknown.

[b]Number positive/number specimens examined.

[c]% tumor cells positive.

[d]Benign cysts included 1 serous cystadenoma, 3 mucinous cystadenomas, 1 simple cyst, 1 serous cystadenocarcinoma of low malignant potential, and 1 mucinous cytadenocarcinoma of low malignant potential. Ovarian carcinomas included serous (7 specimens), mucinous (1) and endometroid (2).

Table 4

Reactivity of MoAbs with normal epithelial cells

MoAb	Positive Tissues
MH99	most or all epithelial cells
MT179	colon, lung, skin, pancreas, breast
MW162	esophagus, stomach, bronchus, uterus, lung, kidney[a], pancreas, thyroid, breast
MW207	bronchus, lung, kidney[a], pancreas, thyroid, uterus, breast
MX35	bronchus, lung, kidney[a], uterus, thyroid

[a]In the kidney, MW207 reacted with proximal tubules, MW162 with Henle's loop and distal tubules, and MX35 with collecting tubules.

Radiolabeled monoclonal antibodies (Mabs) or their fragments are receiving considerable attention as tumor targeting agents for a wide variety of solid tumors (Epenetos et al., 1982; Larson et al., 1983; Mach et al., 1983; Baum et al., 1986; Delaloye et al., 1986; Chatal et al., 1987; Sharkey et al., 1990; Lloyd et al., 1993). Although labeled Mabs are beginning to have an impact in the clinical domain, their specificity and sensitivity for tumor detection still remain to be improved. Radioimmunotargeting with $F(ab')_2$ and Fab fragments of antibodies has generally been favored over the use of the intact Ig due to their shorter biological half-life in the blood and rapid tissue distribution and clearance of the fragments resulting in higher tumor to normal tissue and tumor to blood ratios (Fjeld et al., 1992).

The primary objective of our work was to study the biodistribution and pharmacokinetics of radiolabeled Mab MX-35 in a xenograft model of human ovarian cancer as a preliminary step to clinical studies using this antibody. Mab MX-35 (IgG1) was developed by immunization of mice with ascites and solid tumor ovarian cancer cells (Mattes et al., 1987; Mattes et al., 1989). It reacts strongly and relatively uniformly with 75-80% of ovarian carcinoma samples and with a few normal tissues (Rubin et al., 1989, Rubin et al., 1991). The antigen detected is a cell surface 90,000 dalton, non-secreted glycoprotein (M. Welshinger, B.Y.T. Yin and K.O. Lloyd, unpublished data).

In this study we place special emphasis on radiolabeled $F(ab')_2$ fragments of Mab MX35 as an alternative to the intact antibody. By comparing the tissue distribution of radiolabeled intact IgG with $F(ab')_2$ fragments we demonstrated much better targeting using the fragments. More interestingly, compared with intact antibody, the $F(ab')_2$ fragments gave markedly increased values of absolute tumor uptake of the antibody. This result contrasts with previous results (Sands, 1990; Gerretsen et al., 1991; Molthoff et al., 1992) obtained from comparative experiments.

Materials and Methods

Monoclonal Antibody Production

Monoclonal antibody MX-35, a murine IgG1, was produced from hybridoma ascites grown in BALB/c mice. High titer ascites batches were pooled for purification. MAb MX-35 was purified from the ascites through several steps including removal of lipoprotein by ultracentrifugation at 100,000 g and ammonium sulfate precipitation (50% saturation). Final purification was by protein A-agarose chromatography (Ey et al., 1978).

Preparation of Fragments

Following overnight dialysis of purified MX-35 immunoglobulin in 25mM sodium acetate pH 4.5, 25 μl of pepsin (1.5 mg ml^{-1}; Sigma Chemical Co., St. Louis, MO) was added to the antibody (2 mg in 400 μl) and incubated overnight at 37 C. $F(ab')_2$ fragments were isolated using a kit obtained from Bio Chrom International, Tustin, CA. High yield binding buffer (250 μl) containing 30 μl of anti-pepsin was added to the antibody-pepsin combination. The entire sample was diluted with 220 μl of high yield binding buffer and centrifuged to 2000 g for 15 min and the supernatant was placed on a protein-A-Avidchrome column. The unadsorbed fraction was concentrated using a Centricon 30 unit (Amicon, Beverly, MA) at 1075 g at 4 C. The final antibody concentration obtained was 1.95-2.34 mg ml^{-1} and the overall yield ranged from 63-65%. The identity of the fragments was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Staining with Coomassie blue, revealed bands of 23 kD and 25 kD corresponding to the light and cleaved heavy chains, respectively (Figure 1).

Radiolabeling of Monoclonal Antibodies

Intact specific and control Mab or their $F(ab')_2$ fragments were labeled using chloramine-T method as follows. MAb MX-35 (1 mg) were added to 0.5 ml of 0.15M NaCl in 0.05M phosphate buffer at pH 7.5. One mCi of ^{125}I and 40μl of freshly dissolved chloramine-T (1 mg ml^{-1}) were added on ice. After 10 min the reaction was terminated by addition of 45 μl sodium metabisulfate (1 mg ml^{-1}). The protein was passed through a Sephadex G-25 column (10 ml) and fractions with the highest

radioactivity were pooled. The immunoreactivity of the labeled product was determined by sequential absorptions with an antigen-expressing cell line (OVCAR-3). Between 60 and 75% of the radioactivity was adsorbed for both $F(ab')_2$ fragments and intact antibody MX-35. Percent labeled protein was determined by the TCA precipitation method. For both $F(ab')_2$ fragments and intact antibody incorporation of iodine into protein was 90-95%.

Binding Assays

The method was described elsewhere (Mattes et al., 1989). Briefly, serial 2:3-fold dilutions of radiolabeled intact or $F(ab')_2$ fragments of MAb MX-35 were incubated with 50 μl cell suspension (5 μl packed OVCAR-3 cells) mixed with 100 μl of labeled MAb dilutions ranging from 0.05 $\mu g\ ml^{-1}$ to 20 $\mu g\ ml^{-1}$, for 5 hrs at 4 C. All assays were performed both with (2 mg ml^{-1}) and without a high concentration of unlabeled MAb MX-35. Free MAb was calculated by subtracting bound MAb from total bindable MAb, and the equilibrium association constant Ka was determined by graphical analysis using the method of Scatchard. The off-rates for radiolabeled MAb bound to OVCAR-3 cells were also determined as described by Mattes et al. (1989).

Biodistribution Studies

Athymic nude mice (BALB/c background) were implanted, intraperitoneally (IP), with fragments of the human ovarian cancer cell line, OVCAR-3, obtained by mincing tumor grown in another nude mouse. Visible tumors appeared approximately 3 weeks after injection. Each mouse had tumors of at least 300-500 mg. The expression of MX35 antigen in the tumor was confirmed by immunohistochemistry (Rubin et al. 1989). Mice bearing IP xenografts were randomized to be injected with either 20 μg of ^{125}I-labeled MAb MX-35 [intact or $F(ab')_2$] or the control antibody L6 anti-id 13B (intact or $F(ab')_2$) [kindly provided by Dr. I. Hellstrom, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA] either intraperitoneally or intravenously. The footnote to Table 1 summarizes the number of mice sacrificed at various time points for the eight possible combinations of treatment groups. Tumor tissues and normal tissues from each time point were obtained at the time of sacrifice by dissection. All tumor was recovered from sites within the peritoneal cavity. From

each animal 5 to 9 tumor samples were counted for ^{125}I. These animal experiments were performed in compliance with the relevant national laws relating to the conduct of animal experimentation.

Statistical Analysis

The uptake of antibodies was measured by calculating the % injected dose per gram of tissue. For tumor uptake and serum levels, mono-exponential models for the decay over time were fitted to the data using the method of analysis of covariance. The half-life of MAb serum levels and tumor were estimated using the regression coefficient

estimate B as T ½=log2 / (-B) (Gibaldi & Perrier, 1975).

Tumor specific localization was measured by the data obtained from tumor to blood, tumor to liver and tumor to muscle ratios of % injected dose. The effects of the type and the form of antibody, administration route and time were examined using the method of analysis of variance. Log transformations on the ratios were performed to ensure normality. The localization index (LI) (Beaumier et al., 1985) is defined as the ratio:

$$LI = \frac{\text{\% injected MX-35 } g^{-1} \text{ tumor\% injected control } g^{-1} \text{ tumor}}{\text{\% injected MX-35 } g^{-1} \text{ blood\% injected control } g^{-1} \text{ blood}}$$

A localization index of 1 means that the antibodies have similar standardized uptakes (Moshakis et al., 1981). An index greater than 1 indicates superior uptake of MAb MX-35 over control antibody.

In this study, the control and specific antibodies were not co-administered to the same animals but to two independent experimental groups. The mean tumor to blood ratio (in log scale) for each treatment combination was used to calculate LI. The standard errors of LI were calculated using assumption of normality on the logarithm of tumor to blood ratios.

Results

Characteristics of Radiolabeled Antibody and Fragments

The radiolabeling procedure did not significantly change the immunologic properties of both the whole IgG and $F(ab')_2$ fragments of MAb MX-35. They both retained 60-70% immunological reactivity after radiolabeling. The binding affinities of the intact and $F(ab')_2$ fragments of the antibody for OVCAR-3 cell line were determined by Scatchard analysis. Interestingly, the binding affinity of the intact MAb MX-35 (2.2×10^8 L M^{-1}) was slightly lower than that obtained with $F(ab')_2$ fragments (3.3×10^8 L M^{-1}). The number of binding sites/cell for the two forms was comparable (1.20×10^5 and 1.19×10^5 sites/cell, respectively). The off-rates for bound radiolabeled antibody were 23 hrs and 22 hrs for the intact antibody and fragment, respectively.

Pharmacokinetic Data

Serum clearance rates in 50 animals were determined for the ^{125}I-labeled intact MAb MX-35 and control antibody and in 58 animals for the ^{125}I-labeled $F(ab')_2$ fragments of MAb MX-35 and the control antibody for both IV and IP routes of administration (Table 1). The serum half-life was estimated as 31 hrs for IV and 39 hrs for IP injection for intact MAb MX-35 and 9 hrs for IV, 9.5 hrs for IP injection for the $F(ab')_2$ fragments of MAb MX-35. We could not demonstrate any statistically significant difference in the magnitude of antibody accumulation in tumors and serum between IV and IP routes of administrations for either intact or $F(ab')_2$ fragments of MAb MX-35 or control antibody (all $p > 0.07$) (Table 1). However, the difference between the specific MAb MX-35 and the control antibody L6 anti-id 13B and the difference

between intact and $F(ab')_2$ fragments were significant for serum clearance (Figure 2A) and tumor clearance (Figure 2B). A separate mono-exponential model was thus fitted to each of the four combinations. The models explained the data well as indicated by the correlation estimates. It is clear from Table 1 and Figure 2 that the clearance in serum is consistently faster than that from tumor. For both tumor and serum, the half-life of $F(ab')_2$ fragments are significantly shorter than that of intact MAb (all $p<0.01$). Also the half-life of intact MAb MX-35 was shorter than for control antibody in both tumor and serum (both $p<0.01$). However, the half-life of $F(ab')_2$ fragments of MAb MX-35 in tumor were longer than for the control antibody fragments ($P<0.01$) and in serum there were no differences between the half-lives of $F(ab')_2$ fragments of MAb MX-35 and the control antibody ($P<0.1$). As a result, the clearance of $F(ab')_2$ fragments of MAb MX-35 is relatively faster in serum than in tumor.

Biodistribution Data

Following injection of radiolabeled antibody, peak percent injected dose (mean value) per gram of tumor tissue was 1.6 at 24 hrs for IV and 2.4 at 12 hrs for IP routes of injection for the intact MAb MX-35 (Figure 2b). The corresponding values at 12 hrs were 10.4 for IV and 8.2 for IP administration for $F(ab')_2$ fragments of MAb MX-35. There was no specific localization of the control MAbs in the tumor with peak % injected dose per gram tissue of 0.4 and 2.0 for intact antibody and $F(ab')_2$ fragments, respectively both IV and IP routes of injection.

Accumulation of radioactivity in nomal tissues was consistently lower, for both intact mAb and fragments, than in tumor tissue. Table 2 gives some representative data for

one time point (24 hrs) and data for other time points are presented as tumor/normal tissue ratios in Table 3 and Figure 3. Ratios of tumor to blood, tumor to liver and tumor to muscle had similar patterns. The ratios of MAb MX-35 $F(ab')_2$ fragments increased with time and, in most cases reached a maximum at 61 hrs for both IV and IP routes of injection (Figures 3B, 3D, 3F). The ratios of intact MAb MX-35 also had an increasing trend when administered IV (Figures 3A, 3C, 3E). However, when given IP, neither intact MAb or $F(ab')_2$ fragments of the control antibody had apparent changes over time. The mean ratios at 61 hrs for $F(ab')_2$ fragments and the mean ratios at 72 hrs for intact antibody are tabulated in Table 3. This subset of data was chosen to be close to the peak area within the nature of the study design. Table 3 demonstrated that tumor to normal tissue ratios were much higher for MX-35 $(Fab')_2$ fragments than for any other combination and that the ratios for the IP and IV experimental groups were very similar.

<u>Localization Data</u>

Data for the localization of radiolabeled MAb in tumor relative to blood are shown in Figure 4. The mean LI for $F(ab')_2$ reached a value of 20.6 and 15.2 at 61 hrs for IV and IP injections, respectively, while mean LI for intact MAb was 4.8 and 4.3 for IV and IP injections, respectively with the former value being reached at 72 hrs and the latter at 24 hrs. The differences between IV and IP administrations were not significant except for that of intact antibody at 12 hrs ($p<0.05$) and 24 hrs ($p<0.02$). Generally, $F(ab')_2$ fragments had higher LI than that of intact antibody ($p<0.01$ at 48 hrs).

Discussion

Immunologic binding characteristics of the antibody and the accessibility of the antigenic sites in the tumor are the fundamental basis for the selection of MAbs for MAb-directed radio-immunodiagnosis or radioimmunotherapy of solid tumors. Monoclonal antibodies with desirable characteristics should produce high tumor uptake and accompanying low background activity, i.e., a high target to nontarget ratios, and uniform MAb accumulation within the tumor (Sands et al., 1990; Sharkey et al., 1990; Waldman et al., 1991). The delivery of MAbs into the tumor is influenced by two major determinants; 1) MAb properties such as binding affinity, size of the antibody molecule, dose, immunoreactivity and internalization, and 2) intrinsic tumor properties such as histology, antigen density and homogeneity, vascularity, blood flow, permeability, and size of the tumor (Jain, 1987). In our study, intrinsic tumor properties and MAb properties remained constant between the two experiments except for changes in molecular size of the MAb MX-35.

As the transport of solute molecules into the tumor interstitium is governed by the biological and physicochemical properties of the diffusing molecule, the molecular weight of the administered antibody will strongly influence the rate of passive diffusion across both interstitial and extravascular subcompartments and cell membranes (Jain, 1987). Inaccessibility of antigen-bearing tumor cells to MAbs appear to be a major cause of inhomogeneous distribution of MAb in the tumor. In order to circumvent the problems associated with poor penetration of MAb into tumors, molecules of lower molecular weight are preferable for *in vivo* immunotargeting. Also, an additional

advantage for low molecular-weight MAbs are their rapid clearance by urinary excretion (Bergardat et al., 1970; Buchegger et al., 1986; Endo et al., 1988). Moreover, the fragments lack the Fc region responsible for nonspecific tissue uptake. Radiolabeled fragments are, therefore, usually superior to whole IgG when used for *in vivo* immunotargeting (Buraggi et al., 1985; Andrew et al., 1986; Baum et al., 1986; Andrew et al., 1988). Although these advantages hold true for both Fab and $F(ab')_2$, reports dealing with studies on absolute tumor uptake of whole IgG, Fab and $F(ab')_2$ often show a decrease in tumor uptake of fragments when compared to whole IgG, most likely the result of increased clearance from the blood and a decreased affinity inherent in the generation of fragments (Buchegger et al., 1986; Colapinto et al., 1988; Endo et al., 1988); Wahl et al., 1983). Intact IgG usually gives the highest tumor uptake but this virtue may be overshadowed by high background levels of radioactivity. The renal clearance of whole IgG is relatively slow due to its high molecular weight, whereas fragments are rapidly cleared, thereby improving the tumor/background ratio.

In our investigation, we compared the characteristics of whole antibody MX-35 and its $F(ab')_2$ fragments with regard to biodistribution in nude mice bearing OVCAR-3 xenografts. We noted significant differences in biodistribution between labeled $F(ab')_2$ fragments and whole antibody MX-35, even though the immunoreactive fraction of the labeled antibody was very similar for both. Tumor to normal organ ratios were much higher for $F(ab')_2$ fragments as compared to IgG. Additionally, tumor to blood ratios for the $F(ab')_2$ fragments were approximately 7 times as high at 61 hrs as for IgG at

72 hrs or later (152 hrs) which is a similar finding to previous results obtained with intact MAbs and their fragments (Molthoff et al., 1992; Gerretsen et al., 1991). Interestingly, there was an approximately 5-fold increase in % injected dose per gram with $F(ab')_2$ fragments as compared to whole antibody resulting in a higher localization index (i.e., a higher specific/nonspecific tumor ratio). Our findings contrast with previous reports that have suggested that intact IgG's produce the highest levels of tumor uptake, while fragments produced significantly lower values. Molthoff et al. (1992) reported maximum absolute tumor uptake for intact IgG ranging from 8.5 to 17.7% injected dose per gram for antibody whereas for the respective $F(ab')_2$ fragments the maximum values were 5.2% to 10% injected dose per gram. In the same context, Gerretsen et al. (1992) reported a mean tumor uptake as 14% for whole IgG and 7.2% for $F(ab')_2$ fragments of the same antibody E48. However, in this particular study the investigators did not determine the binding affinities for the fragments. In a clinical study of mAb MOV18 in ovarian cancer patients, Buist et al. (1993) reported %ID/kg of 6.2 and 0.9 for intact IgG and $F(ab')_2$, respectively. The basis for the superior tumor uptake by fragments is in our study not clear. The effect, could be due to the slightly higher affinity shown by the fragments. It is also possible that the number of effective binding sites in the tumor is higher with $F(ab')_2$ fragments than with intact IgG due to better accessibility to the cell surface antigen as a consequence of reduced molecular size. Less surface area is occupied by the smaller $F(ab')_2$ molecules (Molthoff et al. 1992) which might escape a transportation barrier to which the intact antibody is vulnerable. Nevertheless, the number of

binding sites/cell measured *in vitro* with OVCAR-3 cells was very similar for both forms of the MAb MX-35. Also, the off-rates for the cell-bound whole IgG and the fragment are very similar. Another possibility is that the intact MAb may be reacting with Fc receptors in tissues or in blood cells, although this is unlikely as mouse IgG_1 antibodies such as MAb MX-35, do not show this property. The explanation for the superior properties of the fragment will be the subject of further studies.

In summary, the use of $F(ab')_2$ fragments of MAb MX-35 strongly improved absolute tumor uptake of the MAb when compared directly with intact MAb MX-35. An on-going clinical study in patients with epithelial ovarian cancer on the localization of ^{125}I-labeled whole MX-35 antibody has demonstrated modest accumulation (mean %ID g^{-1} = 1.08×10^{-3} at 7 days) of the antibody in tumors (Rubin et al., 1993). These data are comparable to the values noted in the present animal study for whole antibody. This study therefore provides a rationale for a clinical study in patients with epithelial ovarian cancer patients using radiolabeled $F(ab')_2$ fragments.

References

Andrew S. M., Pimm M. V., Perkins A. C. and Baldwin R. W. (1986) Comparative imaging and biodistribution studies with an anti-CEA monoclonal antibody and its $F(ab')_2$ and Fab fragments in mice with colon carcinoma xenografts. Eur. J. Nucl. Med. **12**, 168-175.

Andrew S. M., Perkins A. C., Pimm M. V. and Baldwin R. W. (1988) A comparison of iodine and indium labelled anti CEA intact antibody, $F(ab')_2$ and Fab fragments by imaging tumour xenografts. Eur. J. Nucl. Med. **13**, 598-604.

Baum, R. P., Maul F. D., Senekowsch R., Lorenz M., Hottenrott C., Hopp J., Standke R., Chatal J. F., Saccavini J. C., Kriegel H. and Hor G. (1986) Radioimmuno-scintigraphy of CA 19-9/CEA producing tumors using I-131 labelled $F(ab')_2$ fragments of monoclonal antibodies (19-9/anti CEA radioimmunococktail). In Nuclear Medicine in Clinical Oncology (Edited by Winkler C.), pp.197. Springer, Berlin.

Beaumier P. L., Krohn K. A., Carrasquillo J. A., Eary J., Hellstrom I., Hellstrom K. E., Nelp W. B. and Larson S. M. (1985) Melanoma localization in nude mice with monoclonal Fab against p97. J. Nucl. Med. **26**, 1172-1179.

Bergard I. (1970) Plasma proteins in normal urine. In Proteins in Normal and Pathological Urine (Edited by Manuel Y., Revillard J. P. and Betuel H.), pp. 7-19. Karger, Basel, NY.

Buchegger F., Mach J.-P., Leonnard P. and Carrel S. (1986) Selective tumor localization of radiolabeled anti-human melanoma monoclonal antibody fragment demonstrated in the nude mouse mode. Cancer **58**, 655-662.

Buist M. R., Kenemans P., den Hollander W., Vermorken J. B., Molthoff C. J. M., Burger C. W., Helmerhorst T. J. M., Baak J. P. A., and Roos J. C. (1993) Kinetics and tissue distribution of the radiolabeled chimeric monoclonal antibody MOv18 IgG and $F(ab')_2$ fragments in ovarian carcinoma patients. Cancer Res. **53**, 5413-5418.

Buraggi G. L., Callegaro L., Mariani G., Turrin A., Cascinelli N., Attili A., Bomberdieri E., Terno G., Plassio G., Dovis M., Mazzuca N., Natali P. G., Scassellati G. A., Rosa U. and Ferrone S. (1985) Imaging with I-131-labeled monoclonal antibodies to a high-molecular-weight melanoma-associated antigen in patients with melanoma:efficacy of whole immunoglobulin and its $F(ab')_2$ fragments. Cancer Res. **45**, 3378-3387.

Chatal J.-F., Fumoleau P., Saccavini J.-C., Thedrez P., Curtet C., Bianco-Arco A., Chetanneau A., Peltier P., Kremer M. and Guillard Y. (1987) Immunoscintigraphy of recurrences of gynecologic carcinomas. J. Nucl. Med. **28**, 1807-1819.

Colapinto E. V., Humphrey P. A., Zalutsky M. R., Groothuis D. R., Friedman H. S., deTribolet N., Carrel S. and Bigner D. D. (1988) Comparative localization of murine monoclonal antibody Mel-14 $F(ab')_2$ fragment and whole IgG2a in human glioma xenografts. Cancer Res. **48**, 5701-5707.

Delaloye B., Bischof-Delaloye A., Buchegger F., Von Fliedner V., Grob J.-P., Volant J.-C. Pettavel J. and Mach J.-P. (1986) Detection of colorectal carcinoma by emission-computerized tomography after injection of ^{123}I-labeled Fab or $F(ab')_2$ fragments from monoclonal anti-carcinoembryonic antigen antibodies. J. Clin. Invest.

77, 301-311.

Endo K., Kamma H. and Ogata T. (1988) Radiolabeled monoclonal antibody 15 and its fragments for localization and imaging of xenografts of human lung cancer. J. Natl. Cancer Inst. **80**, 835-842.

Epenetos A. A., Britton K. E., Mather S., Shephard J., Granowska M., Taylor-Papadimitriou J., Nimmon C. C., Durban H., Hawkins L. R., Malpas J. S. and Bodmer W. F. (1982) Targeting of iodine 123 labelled tumour associated monoclonal antibodies to ovarian breast and gastrointestinal tumors. Lancet **2**, 994-1004.

Ey P.L. Prowse S. J. and Jenkin C. R. (1978) Isolation of pure IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Biochemistry **15**, 429-436.

Fjeld J. G., Michaelsen T. E. and Nustad K. (1992) The binding parameters of radiolabelled monoclonal $F(ab')_2$ and Fab' fragments relative to immunoglobulin G in reactions with surface-bound antigens. Eur. J. Nucl. Med. **19**, 402-408.

Gerretsen M., Quak J. J., Suh J. S., Van Walsum M., Meijer C. J. L. M., Snow G. B. and Van Dongen G. A. M. S. (1991) Superior localization and imaging of radiolabeled monoclonal antibody E48 $F(ab')_2$ fragment in xenografts of human squamous cell carcinoma of the head and neck of the vulva as compared to monoclonal antibody E48 IgG. Br. J. Cancer **63**, 37-44.

Gibaldi M. and Perrier D. (1975) Pharmacokinetics. Marcel Dekker, Inc, New York.

Jain R. K. (1987) Transport of molecules in the tumor interstitium: a review. Cancer Res. **47**, 3039-3051.

Larson S. M., Carrasquillo J. A., Krohn K. A., Brown J. P., McGuffin R. W., Ferens J. M., Graham M. M., Hill L. D., Beaumier P. L., Hellstrom K. E. and Hellstrom I. (1983) Localization of ^{131}I-labeled p97 specific Fab fragments in human melanoma as a basis for radiotherapy. J. Clin. Invest. **72**, 2101-2114.

Lloyd K. O. (1993) Immunobiology of ovarian epithelial cancer. In Cancer of the Ovary (Edited by Markman M. and Hoskins W. J.), pp. 47-59. Raven Press, Ltd., New York.

Mach J.-P., Chatal J.-F., Lumbroso J.-D., Buchegger F., Forni M., Ritschard J., Berche C., Douillard J.-Y., Carrel S., Herlyn M., Steplewski Z. and Koprowski H. (1983) Tumor localization in patients by radiolabeled monoclonal antibodies against colon carcinoma. Cancer Res. **43**, 5593-5600.

Mattes J. M., Look K., Furukawa K., Pierce V. K., Old L. J., Lewis Jr. J. L., Lloyd K. O. (1987) Mouse monoclonal antibodies to human epithelial differentiation antigen expressed on the surface of ovarian carcinoma ascites cells. Cancer Res. **47**, 6741-6750.

Mattes J. M., Lloyd K. O. and Lewis Jr. J. L. (1989) Binding parameters of monoclonal antibodies reacting with ovarian carcinoma ascites cells. Cancer Immunol. Immunother. **28**, 199-207.

Molthoff C. F. M., Pinedo H. M., Schluper H. M. M., Nijman H. W. and Boven E. (1992). Comparison of the pharmacokinetics, biodistribution and dosimetry of monoclonal antibodies OC-125, OV-TL3 and 139H2 as IgG and F(ab')$_2$ fragments in experimental ovarian cancer. Br. J. Cancer **65**, 677-683.

Moshakis V., McIlhinney R. A. J., Raghavan D. and Neville A. M. (1981) Localization of human tumour xenografts after IV administration of radiolabelled monoclonal antibodies. Br. J. Cancer **44**, 91-99.

Rubin S. C., Finstad C. L., Hoskins W. J., Federici M. G., Lloyd, K. O., and Lewis Jr. J. L. (1989) A longitudinal study of antigen expression in epithelial ovarian cancer. Gynecol. Oncol. **34**, 389-394.

Rubin S. C., Finstad C. L., Hoskins W. J., Provencher D., Federici M. G., Lloyd K. O. and Lewis Jr. J. L. (1991). Analysis of antigen expression at multiple tumor sites n epithelial ovarian cancer. Am. J. Obstet. Gynecol. **164**, 558-563.

Rubin S. C., Kostakoglu L., Federici M. G., Finstad C. L., Lloyd K. O., Larson S. M., Hoskins W. J. (1993). Clinical trial of pharmacokinetics, biodistribution and intraoperative radioimmunodetection of radiolabeled monoclonal antibody (MAb) MX-35 in epithelial ovarian cancer. Gynecol. Oncol. **49**, 116-117.

Sands H. (1990) Experimental studies of radioimmunodetection of cancer; An overview. Cancer Res. (Suppl.) **50**, 809s-813s.

Sharkey R. M., Blumenthal R. D., Hansen H. J. and Goldenberg D. M. (1990) Biological considerations for radioimmunotherapy. Cancer Res. (Suppl.) **50**, 964s-969s.

Wahl R. L., Parker C. W. and Philpott G. W. (1983) Improved radioimaging and tumor localization with monoclonal $F(ab')_2$. J. Nucl. Med. **24**, 316-325.

Waldman T. A. (1991). Monoclonal antibodies in diagnosis and therapy. Science (Washington, D.C.) **252**, 1657-1662.

Table 1. **Comparison of Serum and Tumor Half Lives of Whole Antibody and Antibody Fragments**

MAb[a]	Route	Antibody half-life (hours) Serum	Tumor
MX-35 intact	IV	30.8	42.0
	IP	39.3	36.7
L6[c] intact	IV	ND[b]	ND
	IP	77.5	141.6
MX-35 $F(ab')_2$	IV	9.0	16.6
	IP	9.5	16.6
L6[c] $F(ab')_2$	IV	10.9	12.0
	IP	10.1	12.1

[a] For intact and $F(ab')_2$ forms of MX-35, 3 or 4 mice were used for each time point (IV or IP) except for the 61 and 68 hr time points with the $F(ab')_2$ fragments when 2 mice were used. For the control MAb, 2 mice were used for each time point except for the 24, 61 and 68 hr IP points when one mouse was used.

[b] Not determined. The antibody level essentially did not decline during this time period. The slope was close to 0.

[c] MAb L6 anti-id 13B was used as control.

Table 3. **Summary of Biodistribution Data of the Radiolabeled Antibodies**

Monoclonal Antibody	Route	Ratios Tumor:Blood	Tumor:Muscle	Tumor:Liver
MX-35 intact	IV	1.9	12.3	6.3
	IP	1.4	9.0	3.7
L6[c] intact	IV	0.4	2.8	1.6
	IP	0.5	2.9	2.0
MX-35 $F(ab')_2$	IV	12.9	49.2	9.3
	IP	11.5	64.2	28.4
L6[c] $F(ab')_2$	IV	0.6	5.0	2.0
	IP	0.8	5.1	2.0

All of the above means were taken in log scale. The mean ratios are tabulated for intact antibody at 72 hrs and for $F(ab')_2$ fragments at 61 hrs. See Table 1 for number of mice used in each experimental group.

[c] MAb L6 anti-id 13B was used as control.

Brief Description of the Figures

Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis under reducing conditions of MAb MX-35 (lanes a, b, c) and control MAb L6 anti-id 13B (lanes d, e, f) before enzyme treatment (lanes a, d); after pepsin digestion overnight at 37 C (lanes b, e); and following passage through a protein A column to remove undigested intact IgG (lanes c, f). The gel bands are visualized by Coomassie blue staining. Bands of Mr 50,000, Mr 25,000 and Mr 23,000 correspond to the heavy chains (hc), heavy chain fragments (hc-f), and light chains (lc), respectively.

Figure 2: Mean percentages of injected radioactivity in blood (Panel A) and in tumor (Panel B) showing clearance of intact IgG and $F(ab')_2$ fragments of MAb MX-35 and control MAb L6 anti-id 13B. For intact IgG N=6 for all time points. For $F(ab')_2$ fragments of MAb MX-35 N=7 at 12, 24, and 48 hours, N=8 at 38 hours and N=5 at 61 and 68 hours. The experimental groups (IP and IV combined) are distinguished as follows: intact MAb MX-35 (black dashed line); MAb MX-35 $F(ab')_2$ fragments (black solid line); control intact MAb L6 (stippled dashed line); and control MAb L6 $F(ab')_2$ fragments (stippled solid line). Vertical bars indicate range or +/-2 standard errors.

Figure 3: Biodistribution data indicating tumor to blood ratios (A, B), tumor to muscle ratios (C, D); and tumor to liver ratios (E, F) and comparing intact IgG (A, C, E) and $F(ab')_2$ fragments (B, D, F). All groups

consisted of 3 or 4 mice per time point. The ratios for $F(ab')_2$ fragments continued to increase up to 68 hours whereas intact antibody did not show an apparent change over time. The experimental groups are distinguished as follows: intact or fragmented MAb MX-35 by IP injection (black solid line) or by IV injection (black dashed line); intact or fragmented control MAb L6 by IP injection (stippled solid line) or by IV injection (stippled dashed line). Vertical bars indicate range or +/- 2 standard errors.

Figure 4: Comparison of tumor localization for intact IgG and $F(ab')_2$ fragments. The localization index reflects the ratio of activities in tumor of "specific" intact MAb MX-35 and $F(ab')_2$ fragments over "nonspecific" control intact MAb L6 and $F(ab')_2$ fragments and corrected for blood activity at the same time. There was much more rapid development of specific uptake with $F(ab')_2$ fragments of MAb MX-35 as compared to the intact MAb MX-35. The experimental groups are distinguished as follows: intact IgG by IP injection (stippled dashed line) or by IV injection (stippled solid line); $F(ab')_2$ fragments by IP injection (black dashed line) or by IV injection (black solid line). Vertical bars indicate range or +/-2 standard errors.

Synopsis:

This is a Phase I dose escalation study using ^{131}I-MX35 F(ab')$_2$ (2 mg/dose) for treatment of patients with low volume (≤ 0.5 cm diameter, documented at post-chemotherapy laparotomy) MX35 antigen-expressing (≥ 25% of cells in an average high power field) ovarian carcinoma. Three patients per dose level will be followed for up to 8 weeks with biochemical and hematologic tests for toxicity, and CA-125 estimations for response. The first cohort of patients will receive 30 mCi ^{131}I labeled to 2 mg MX35 F(ab')$_2$ antibody. Subsequent dose escalation will be in 30 mCi ^{131}I (labeled to 2 mg MX35 F(ab')$_2$). Patients will be treated as outpatients in the Adult Day Hospital. Doses will be administered at intervals of 4-5 days. Patients at the first dose level will receive one outpatient infusion. Patients at subsequent dose levels will receive repeat outpatient infusions at least 4 days after the prior infusion, with total patient radiation levels to be ≤ 5 mR/h at 1 meter at completion of infusion. All infusions will be completed within 14 days, to obviate/minimize anti-murine antibody responses *during* infusion. In the absence of disease progression and after recovery from toxicity patients may be re-treated beginning 8 weeks after the prior infusion, for a total of not more than three treatments.

A Maximum Tolerated Dose (MTD) will be determined, defined as the highest dose at which not more than a third of the patients have Grade IV hematopoietic or Grade III or greater non-hematopoietic toxicity. Serologically evaluable patients will be studied for possible responses.

TABLE OF CONTENTS

1 · OBJECTIVE:

To determine the safety, toxicity and maximum tolerated dose (MTD) of ^{131}I-MX35 $F(ab')_2$ administered by intraperitoneal (ip) injection to patients with residual ovarian cancer.

2 BACKGROUND AND RATIONALE:

2.1 Current therapy of ovarian carcinoma.

Epithelial ovarian cancer (EOC) is the fifth most common malignancy and the leading cause of death from gynecologic malignancies among women in the United States, with an annual incidence of 26,000 cases. The disease predominantly affects postmenopausal women in their sixth decade and accounts for approximately 15,000 deaths each year (1). Seventy percent of women present with advanced disease in which the tumor has spread to the peritoneal surface of the upper abdomen. Extensive intraabdominal disease is difficult to eradicate completely by surgery, and many patients have only a partial response to postoperative chemotherapy.

A tumor-associated antigen, CA-125, found in serum, is used as a disease marker. Serum CA-125 level is elevated in approximately 80% of patients with EOC and is a useful indicator of disease status during and after therapy in those patients. Adequate and complete surgical intervention is mandatory primary therapy for ovarian carcinoma, permitting precise staging, accurate diagnosis and optimal cytoreduction. This is usually followed by combination chemotherapy that includes a platinum analogue - either cisplatin or carboplatin. A platinum-containing regimen forms the cornerstone of first-line treatment of advanced EOC. Chemotherapy is most effective in patients who have undergone maximal cytoreductive surgery or who present with low volume disease. Preliminary results strongly suggest that paclitaxel (Taxol) is very active in EOC. Six cycles of chemotherapy have become the standard and yield clinical response rates of approximately 60% to 70% and 5-year survivals of 10% to 20% (2).

A combination of CA-125 testing and general physical and pelvic examinations have been shown to detect progression of disease in 90% of patients with recurrent EOC. However, benign disease can give false positive predictions in up to 10% of such patients. Serial rise in CA-125 levels > 25% have been shown in several studies to predict progression with almost 100% specificity (3,4,5). Routine radiological examinations (CT, MRI) have not been shown to improve detection of recurrence, and these studies are not routinely used in evaluation.

Currently available salvage therapy for the majority of patients with relapsed EOC is not curative. Paclitaxel is the most active single agent for treatment of relapse, even in patients refractory to platinum. It has a general response rate of approximately 35% (2). There is no evidence at present that early reintroduction of chemotherapy is of any survival benefit, or that searching for the site of relapse will result in surgery that can improve survival. Repeat

surgical debulking may benefit a subset of highly selected patients with relapse, and serves to palliate complications such as bowel obstruction, to improve the quality of life. When a patient relapses for the second time, even with low-volume disease, there is almost no possibility of cure.

There is thus a need of development for alternative treatment strategies in patients who demonstrate residual disease at the time of repeat laparotomy (carried out after initial debulking and chemotherapy).

2.1.1 **Intraperitoneal radiolabeled antibody therapy in EOC.**

Ovarian cancer remains largely confined to the peritoneal cavity during much of its natural history. This has encouraged intracavitary use of a variety of therapeutic radiopharmaceuticals, such as colloidal ^{32}P chromic phosphate, for advanced disease. Radiolabeled antibodies were first introduced by intracavitary injection by Epenetos *et al* (6). They claimed that a higher concentration of radioactivity could be achieved in tumor after local instillation than by systemic administration. Preliminary studies in ovarian cancer, using first ^{131}I- and then ^{90}Y- labeled HMFG antibodies showed promise (7, 8). Patients with second relapse showed longer disease-free survival than historical controls (15/16 free of disease @ 5 years) after treatment with ^{90}Y-HMFG. The group also described the use of a chelate, administered to clear the blood of free ^{90}Y, as a way to improve the MTD by a factor of 3 (8). Toxicity was almost exclusively hematopoietic.

A variety of other investigators have subsequently shown the benefits of IP injection of radiolabeled antibody in ovarian cancer, with response rates of >50% even in advanced disease (9, 10, 11). However, most series, with the exception of the studies by Epenetos *et al*, were in patients with bulky disease who had failed prior chemotherapy, and responses were of short duration.

Radiolabeled antibodies are most effective in small volume disease and the ideal trial design would involve relapsed patients with small volume disease. Complete responses should be possible in this setting.

In preliminary IP trials with ^{131}I-B72.3 (12), radioactivity uptake in peritoneal implants was shown to be considerably better by the IP route than by the IV route, as opposed to more vascularized tumors. Concentration was excellent and tumoricidal doses of 60-100 Gy were predicted from the biodistribution (13).

In a Phase I dose escalation IP trial with ^{131}I-B72.3, doses of up to 125 mCi were well tolerated. One patient, with a gastrointestinal primary and peritoneal implants, is now free of disease 8 years after treatment with 100 mCi ^{131}I-B72.3 IP (A. Raubitschek, personal communication, 1995).

2.2 **Antibody Background**

2.2.1 Development and specificity

Monoclonal antibody MX35 is a murine IgG_1 that was developed in the laboratory of Dr. Kenneth O. Lloyd at the Sloan-Kettering Institute (14).

Fresh ovarian carcinoma specimens, including two solid tumors and two samples of ascites cells, were used as the immunogens. The antibody has been tested for reactivity on a wide variety of cryostat sections of normal and malignant human tissues, tissue culture cell lines, and ABO blood group-related antigen preparations, and biochemical characterization has been performed. In fresh frozen sections, MX35 was detected in epithelial cells of the normal bronchus, lung, sweat glands, kidney collecting ducts, thyroid, fallopian tube, cervix and uterus. All other tissues examined, including normal mesothelium (peritoneum) were negative. MX35 was detected in sections of 420 of 460 fresh frozen ovarian carcinomas. In tissue culture, MX35 was detected on 3 of 8 ovarian cancer cell lines.

2.2.2 **MX35 IgG clinical trial:** In a clinical trial of intact MX35 administered intravenously or intraperitoneally to ovarian cancer patients conducted at Memorial Sloan-Kettering (IRB 89-118), twenty-two patients with advanced ovarian cancer received ^{125}I or ^{131}I-labeled MX 35 in doses of 2, 10, or 20 mg administered by intravenous (IV) or intraperitoneal (IP) injection. All patients underwent laparotomy at 7 to 20 days following MAb injection to assess tumor distribution, obtain biopsies of tumor and normal tissue, and evaluate the use of an intraoperative hand held gamma-detecting device. Following IV injection, serum MAb half life was 36 hours. Tumor biopsies obtained at surgery showed MAb accumulation of from 3.8×10^{-5} to 4.0×10^{-4} % injected dose/gram of tissue . There was no correlation between absolute MAb accumulation in tumor and MAb dose administered. There was a general, though not statistically significant, relationship between MAb uptake and the level of immunohistochemical expression of the MX 35 antigen in a given patient's tumor. Regression analysis showed a correlation between MAb accumulation and the interval between MAb injection and surgery ($P = 0.008$). Specific localization of MAb in tumor was demonstrated by tumor:normal tissue ratios ranging from 2.3:1 to 34:1 (mean 9.6:1). The tumor:normal tissue ratios were not significantly related to MAb dose, the level of immunohistochemical antigen expression, or the interval between MAb injection and surgery. Due to the relatively long serum half life, mean tumor:serum ratios were only 1.66 following IV injection. This ratio did not correlate with MAb dose, days from injection, or antigen expression. There was an excellent correlation ($P = <0.0001$) between MAb uptake, as measured by the intraoperative hand-held gamma counter, and direct gamma counting of excised tissues. No toxicity was seen in this trial (15). It is hoped that the use of MX 35 F(Ab')2 fragments will speed clearance of circulating and non-specifically bound antibody and thus result in improved tumor to

blood and tumor to normal tissue ratios.

2.2.3 **Pre-Clinical MX35 F(Ab')$_2$ Fragment Data** In a nude mouse model of human ovarian cancer the biodistribution of radiolabeled intact MX 35 was compared to that of radiolabeled MX35 F(Ab')$_2$ fragments (Kostakoglu, L., and Rubin, S., unpublished data, 1995). Immunodeficient mice were inoculated IP with the human ovarian cancer cell line OVCAR-3. After establishment of tumor growth the animals were injected either IP or IV with 131-I labeled intact MX35 or F(Ab')$_2$ fragments. The nonreactive antibody anti-id L6 was used as a negative control for the intact antibody and fragments. Animals were sacrificed at various time points between 1 and 8 days for direct gamma counting to determine blood, tumor, and normal tissue levels of specific and control antibody. Whole body gamma counting was performed to assess antibody clearance. Serum T½ was approximately 30 hrs for the intact antibody and 12 hours for the F(ab')$_2$ fragments, with no significant difference by route of injection . Specific localization of intact MX35 to tumor was demonstrated for both routes of injection, with peak tumor to normal tissue ratios of 12:1 and 14:1 following IP and IV injection respectively . Peak ratios for the F(ab')$_2$ fragments were 85 and 33 by the IP and IV routes respectively. There was no evidence for specific localization of either control MAb. Absolute accumulation of intact antibody in tumor was in the range of 0.5 to 3.0 % of injected dose/gm, compared to 7 to 10% for the F(Ab')$_2$ fragments Tumor to blood ratios, which never rose above one using intact antibody, reached a range of 12 to 17 at 61 hours following IV or IP injection of F(Ab')$_2$ fragments. These data suggest that the use of fragments will accelerate clearance of antibody from the circulation, resulting in increased tumor to normal tissue and tumor to blood ratios.

2.2.4 **Clinical trial with MX 35 F(ab')$_2$.**

A pre-surgical trial with radioiodinated MX 35 F(ab')$_2$ was recently carried out at this Center (IRB #94-13). Five patients received 2mg and one patient received 10 mg MX 35 F(ab')$_2$ by the IV route. Two patients received 2 mg MX 35 F(ab')$_2$ IP. The study revealed that there was specific targeting of radioantibody to antigen-positive tumor cells, as determined by contiguous slice comparison of histopathology, immunochemistry, and autoradiography. Most "tumor nodules" consisted largely of fibrous tissue with clusters of tumor cells within; conventional parameters of antibody targeting such as tumor:serum ratios were not therefore useful. Based on this small sample size, it appears that MX 35 F(ab')$_2$ tumor uptake is not dose-dependent, and that tumor uptake is greater when the antibody is administered IP.

A major constraint precluding completion of this trial was accrual. Most patients were unwilling to participate because they would be unable

to receive any therapeutic antibody.

2.2.5 **Iodine-131.**

Iodine-131 has been used in the therapy of differentiated thyroid cancer for several decades. The biodistribution and kinetics of free iodide are well known; uptake of radioactive iodine by thyroid and stomach can be effectively blocked by oral administration of stable iodide; radioiodine can be stably conjugated to antibody; and experience with systemic administration of radioiodinated antibodies in solid tumors, lymphomas and leukemias is considerable and growing.

Its relatively low (E_{max} 0.6 MeV) energy beta-minus (β^-) emissions may prove optimal for small volume disease; its gamma emission (364 KeV) will permit external imaging.

2.3 **The Role of intraperitoneal therapy in ovarian cancer**

In spite of the high responses achieved with platinum-based therapy in ovarian cancer, the number of patients achieving a pathologically documented negative second-look operation remains in the 20-25% range. For those patients achieving a complete response to front-line therapy, the use of intraperitoneal chemotherapy is potentially beneficial, particularly in the situation where tumor is confined to the peritoneal cavity, an extravascular space, thus allowing the achievement of higher drug concentrations to reach the tumor than achievable by intravenous therapy alone.

3 **Summary:**

The rationale for the use of ^{131}I-MX35 F(ab')$_2$ in ovarian cancer is based on the localization of the antibody studied by autoradiography in a current clinical trial (IRB #94-13) and on uniform distribution of antigen in expressing ovarian tumors. The rationale for intraperitoneal administration is based on the observation that the disease is largely confined to the peritoneal cavity and is therefore ideally suited for intracavitary therapy. Delivery of the radiolabeled antibody into the peritoneal cavity allows greater concentration in tumors while reducing radiation exposure to normal tissue, particularly bone marrow. Iodine-131 is the preferred radionuclide as it can be attached easily to the antibody; there is little non-specific binding; its beta-minus emission characteristics are optimal for radioimmunotherapy; because prior clinical experience has shown that the radiolabeled antibody targets to tumor *in vivo*; and, given the proposed dosing schedule, outpatient therapy will be feasible.

Tumor will be obtained for immunohistochemical determination of antigen expression. Only those patients that are antigen-positive (> 25% of tumor cells positive in an average high power field), will be eligible for the trial, so that therapeutic benefit may be expected. Patients will be treated between 4 and 6 weeks after repeat-look surgery with the appropriate dose of ^{131}I-MX35 F(ab')$_2$ ip. They will then be observed for toxicity, especially bone marrow (*vide infra*).

4 MONOCLONAL ANTIBODY MX35 F(ab')$_2$

4.1 Supply

Monoclonal antibody MX35 in a form satisfactory for human administration will be produced at the Sloan-Kettering Laboratories under the supervision of Dr. Lloyd J. Old.

4.2 Production

Hybridoma ascites is produced in pristane treated, irradiated (BALB/c x C57BL/6) FI mice. High titer ascites batches are pooled for purification. Ascites is tested for murine virus (MAP, MSSV, EdIM, thymic virus, LDH, MuLV [complete] LCM). Only ascites batches negative in these tests are pooled for purification.

4.3 Purification

MX35 will be purified from ascites through several steps including removal of lipoprotein by ultracentrifugation at 100,000 g, ammonium sulfate precipitation, and chromatographic fractionation. Purified MX35 will be tested for absence of DNA and characterized by electrophoresis.

4.4 Production of MX 35 F(Ab')$_2$ fragments

MX 35 F(Ab')$_2$ fragments will be produced in the laboratory of Dr. Kenneth O. Lloyd by pepsin digestion of intact antibody, which cleaves the IgG molecule at the F_c region, generating an F(Ab')$_2$ fragment of approximately 100,000 daltons, and various smaller F_c fragments. The protease is subsequently inactivated and complexed with an anti-pepsin antibody. The mixture is then passed through a protein A cartridge, which removes the complexed pepsin and undigested IgG. The F(Ab')$_2$ fragments are collected in the flow through fractions, and the smaller Fc fragments (less then 50,000 daltons) that may co-elute are removed by dialysis using a 50,000 MW cutoff membrane.

The product is characterized by SDS-polyacrylamide gel electrophoresis for its biochemical purity. It is tested for residual pepsin by Western blotting with an anti-pepsin antibody. It is also tested for the presence of blood group A antigen (a possible contaminant of pepsin) with an anti-A antibody.

4.5 Preclinical Safety Testing

Purified MX 35 F(AB')2 fragments will undergo safety testing including the pyrogenicity assay in rabbits, sterility testing, and general safety tests in animals. Only batches of MX 35 F(AB')2 fragments that satisfy the purity and safety criteria outlined above (in accordance with current FDA guidelines) will be used in the proposed study.

4.6 Labeling of MX35 F(ab')$_2$ with ^{131}I

2 mg MX 35 F(ab')$_2$ will be labeled with 30 mCi ^{131}I. Sterile technique and pyrogen-free glassware are used in all labeling steps. To the requisite amount of MAb F(AB')2, at a pH of 7.4, will be added the requisite quantity of ^{131}I. 50 µl of freshly prepared chloramine-T (2 µg/µl) will be added and the solution incubated at room temperature for 2 minutes after which the reaction will be stopped by addition of 50 µl of sodium metabisulfite (10 µg/µl) and transferred to a 10 ml Biogel P6 column (50-100 mesh). Fractions with the highest amount of radioactivity are pooled and filtered through a .22 µ filter. Immunoreactivity of

labeled antibody will be tested by sequential absorptive assay with an antigen-expressing cell line.

The exact amount of radioiodinated MAb $F(ab')_2$ to be injected will be 2.0 mg. The amount of ^{131}I in the final product will be measured in a dose calibrator prior to injection. This will enable future pharmacokinetic and biodistribution quantitation.

4.6.1 Route of administration: Doses will be administered intraperitoneally, in 100 mL 5% human serum albumin, over 30 minutes. Up to 2 liters of normal saline will subsequently be administered intraperitoneally to aid distribution.

5 PROTECTION OF HUMAN SUBJECTS

5.1 A Human AntiMouse Antibody response is invariable following administration of mouse antibody to patients with solid tumors. This may interfere with serologic tests including CA-125. Potential risks to intraperitoneal radioimmunotherapy (RIT) include myelosuppression. No dose-limiting non-hematologic toxicity or side-effects have been noted in therapy trials with up to 75 mCi/m^2 IV ^{131}I.

5.2 Potential benefits include increased time to progression.

5.3 Thrombocytopenia has been the dose-limiting toxicity with other RIT. The patient's platelet count will be monitored frequently (*vide infra*) and platelet transfusions given per hospital guidelines when necessary. Neutropenia will be managed with G-CSF administrations pre guidelines. All patient material and data that is sent outside the institution will be identified by the hospital MRN number; the patient's identity will otherwise not be disclosed.

5.4 Since the patient has failed standard surgery/chemotherapy, the RIT may be of benefit, with otherwise minimal side-effects. Estimation of therapeutic dose should moreover permit Phase II trials in patients, with potential efficacy.

5.5 There is no standard therapy available for patients who will be eligible to enter this study. Other experimental therapies include other IP chemotherapy regimens, as well as experimental systemic chemotherapy.

5.6 The patients will be responsible for standard tests including CBC, screening profiles, and CA-125 estimation. The patient will also be responsible for physician charges. The interferon-gamma, radiolabeled antibody and all *in vitro* studies consequent to the therapy will be offered without charge, unless the radiopharmaceutical and/or the IFN-γ is approved for use for this indication by the FDA.

6 PATIENT ELIGIBILITY

6.1 Patients must have histologically confirmed ovarian carcinoma.

6.2 All patients with ovarian carcinoma scheduled to undergo surgical re-assessment after chemotherapy will be eligible.

6.3 Patients with minimal residual disease after standard surgical debulking

and chemotherapy, who are eligible for intraperitoneal chemotherapy, will be eligible for entry into the protocol.

6.4 Patients must be willing to receive further intraperitoneal chemotherapy if eligible.

6.5 Only those patients who fulfil the following criteria will be eligible for the study:

6.5.1 MX35 antigen tumor expression $\geq$ 25% (ie. at least 25% of cells in an average high-power field should be reactive with MX35 on immunohistochemistry).

6.5.2 Residual disease after surgical assessment $\leq$ 0.5 cm diameter in a patient deemed to be a good candidate for intraperitoneal therapy.

6.5.3 Uneventful placement of an indwelling intraperitoneal catheter.

6.6 Karnofsky performance status of $\geq$ 60%.

6.7 Patients must have adequate organ function as defined by:

6.7.1 WBC $\geq$ 3000/mm^3, platelet count $\geq$ 100,000/mm^3.

6.7.2 Bilirubin $\leq$ 2.0 mg/100 ml.

6.7.3 Creatinine $\leq$ 2.0 mg/100 ml or creatinine clearance $\geq$ 40 ml/min.

(These tests may be carried out within 2 weeks of starting therapy.)

6.8 No evidence of active infection which requires antibiotic therapy.

6.9 Signed informed consent.

6.10 Patients must be at least 18 years of age.

6.11 Patients must have recovered from the toxicity of any prior therapy, and not received chemotherapy or radiation therapy for at least 4 weeks prior to entry into the trial.

7 EXCLUSION CRITERIA:

7.1 Patients with no evidence of residual disease at surgical assessment are ineligible.

7.2 Patients in whom surgical assessment was carried out > 45 days prior to entry into protocol are ineligible.

7.3 Patients with dense intraperitoneal adhesions preventing adequate intraperitoneal distribution are ineligible.

7.4 MX35 tumor expression < 25% (ie. <25% of cells in an average high-power field reactive with MX35 on immunohistochemistry).

7.5 Concurrent radiotherapy other than ^{131}I-MX35 F(ab')$_2$ is not permitted.

7.6 Patients with any significant intercurrent medical problems which will limit the amount of ^{131}I-MX35 F(ab')$_2$ they can tolerate are ineligible for this Phase I study.

7.7 Clinically significant cardiac disease (New York Heart Association Class III/IV), or severe debilitating pulmonary disease.

7.8 Survival expectancy less than 12 weeks.

7.9 Prior therapy with a murine monoclonal antibody.

7.10 Patients with a history of autoimmune hepatitis or history of autoimmune disease.

8 TREATMENT PLAN

8.1 **Accrual Rate:** The expected annual accrual rate is 20 patients.

8.2 **^{131}I-MX35 F(ab')$_2$ Administration:**

8.2.1 2 mg monoclonal antibody MX35 F(ab')$_2$ will be radiolabeled with ^{131}I. The mass amount of MX35 F(ab')$_2$ will be fixed at 2 mg. The initial dose of ^{131}I will be 30 mCi. Iodine-131 dose will be escalated by repeat infusions. The schema will be as follows:

Dose level	Total Dose of ^{131}I	No. of patients	Days of Injection (Dose of ^{131}I)			
1	30	≥ 3	1 (30)	-	-	-
2	60	≥ 3	1 (30)	4 (30)	-	-
3	90	≥ 3	1 (30)	4 (30)	8 (30)	-
4	120	≥ 3	1 (30)	4 (30)	8 (30)	11(30)

8.2.1.1 In order to estimate kinetics of mAb distribution as well as for dosimetric estimates, 10 mCi Tc-99m HSA will be infused along with the ^{131}I-MX35 F(ab')$_2$. A 2 ml sample of peritoneal fluid will be obtained 15 minutes, 1 hour and 2 hours after completion of the infusion, to allow determination of mAb and ip fluid kinetics. A 2 ml sample of blood will be collected at the same times.

8.2.2 Patients will receive ^{131}I-MX35 F(ab')$_2$ intraperitoneally. Infusion will consist of ^{131}I-MX35 F(ab')$_2$ dose diluted in 100 ml of 5% human serum albumin delivered as a 30 minute continuous intraperitoneal infusion, followed by 900 ml of normal saline. Up to an additional one liter of normal saline will subsequently be infused. The total volume of intraperitoneal fluid administered will not exceed two (2) liters, and will be recorded.

8.2.3 All administrations of ^{131}I-MX35 F(ab')$_2$ will be performed under clinical observation in the Adult Day Hospital. A crash cart with medications for the treatment of anaphylaxis will be immediately available.

8.2.4 Radiation safety precautions will be observed by all personnel as outlined in the appended guidelines. Patients will be kept in the Adult Day Hospital until cleared by a member of the Radiation Safety Service. We do not expect any patient to require radiation isolation.

8.3 Duration of Treatment

8.3.1 Toxicity: Patients will be observed for toxicity for at least eight weeks before dose escalation is carried out.

8.3.1.1 If no Grade 3 or 4 toxicity is observed among the initial three patients placed on a dose level, the dose will be escalated for the successive group of three patients.

8.3.1.2 If one instance of Grade 3 or Grade 4 toxicity is observed among the initial three patients placed on a dose level, three additional patients will be treated at that level. If no further instances of Grade 3 or 4 toxicity are observed, the dose will be escalated for the successive group of three patients.

8.3.1.3 If two instances of Grade 3 or Grade 4 toxicity are observed for a given dose level, three additional patients will be treated at that level. If no further instances of Grade 3 or 4 toxicity are observed, the dose will be determined to be the MTD, and no further dose escalation will be carried out.

8.3.2 **Retreatment:** None. Patients will receive only one course of therapy.

8.3.3 **Further therapy:** After recovery from toxicity if any, and not earlier than 6 weeks or later than 12 weeks after the last dose of ^{131}I-MX35 F(ab')$_2$, they will receive intraperitoneal chemotherapy if eligible.

8.4 **CA-125 and HAMA**

HAMA may interfere with CA-125 measurements. The serum for CA-125 will be batched and at the time of measurement 10µL of 10µg/ml of MX35 will be added to the assay to offset any false elevations of tumor marker evaluation.

8.5 **Imaging**

Gamma camera imaging of all patients will be carried out in the Nuclear Medicine service. Anterior and posterior images will be obtained the day of the first infusion; before and after subsequent infusions; daily until all infusions are complete; and three days after the last infusion. Images will be designed to estimate quantitative distribution of radioactivity in the peritoneal cavity, and to estimate kinetics of radioactivity transfer into the systemic circulation. SPECT imaging of relevant areas will be carried out as deemed necessary. Daily imaging time is not expected to be greater than 2 hours.

8.6 **Whole body counting**

Whole body counts will be obtained whenever the patient is in the Nuclear Medicine service. These counts will be obtained using the standard whole body counting crystal (with, if necessary, an appropriate lead shield) as well as with a radiation survey meter. The former will provide percent retention estimates while the latter will measure mR/h at 1 meter.

8.7 **STUDY PARAMETERS See Appendix A.**

9 CRITERIA FOR REMOVAL FROM STUDY

9.1 Progressive disease after a minimum of 6 weeks as defined in 14.1.5.

9.2 Intercurrent illness which prevents further administration of ^{131}I-MX35 F(ab')$_2$.

9.3 Decision of the patient to withdraw from the study.

9.4 General or specific changes in the patient's condition which render the patient unacceptable for further treatment in the judgment of the investigator.

10 MANAGEMENT OF TOXICITY

10.1 **Hazards and Protection**

10.1.1 **Radiation** ^{131}I-MX35 F(ab')$_2$: The long-term toxicities of intraperitoneal radiolabeled MoAb therapy are not known. Potentially any of the chronic toxicities associated with whole abdominal external beam therapy such as bowel or bladder fibrosis, liver dysfunction or peritonitis with adhesions could occur.

10.1.2 **Radiation Precautions:** See Attached Radiation Safety Procedure Guidelines. Laboratory specimens will be labeled with radioactivity warning labels, and will be transported to the Nuclear Medicine Lab by authorized personnel.

10.1.3 **MoAb MX35 F(ab')$_2$:** There has been minimal toxicity with the clinical administration of murine monoclonal antibodies in over 400 patients studied at this institution. Over 20 patients have received either intact or fragmented MX35 without side-effects. One of these patients (who received 10 mg ^{131}I-MX35 F(ab')$_2$ IV) had an idiosyncratic, transient hyperthyroidism, felt possibly to be due to stable iodine toxicity (Jöd-Basedow Syndrome). No such toxicity has been seen in over 500 patients treated at this institution with comparable doses of stable iodide.

10.1.4 **Management of toxicity from MX35:** Bronchospasm and anaphylaxis are rare complications of murine MoAb infusion. Should such occur, the MoAb infusion will be immediately stopped and epinephrine SC, steroids, respiratory assistance and other resuscitative measures undertaken. No further MoAb will be given to such a patient.

11 REPORTING OF ADVERSE REACTIONS

11.1 Known toxicities from ^{131}I-MX35 F(ab')$_2$

For the purposes of reporting ADRs, the following are considered to be known toxicities of the agent being studied: fever, diarrhea, pancytopenia and peritonitis. Repeated administration of murine MoAbs can result in allergic reactions including serum sickness and anaphylaxis.

11.2 Adverse drug reactions (ADRs) to ^{131}I-MX35 F(ab')$_2$ are to be reported promptly to the Institutional Review Board. The following schema is to be followed: Previously unknown Grade 2 and Grade 3 reactions are to be reported within 10 working days. Grade 4 reactions and patient deaths while on treatment are to be reported within 24 hours. A written report is to follow within 10 working days.

12 ADEQUACY OF TRIAL:

12.1 Patients will receive intraperitoneal chemotherapy after recovery from toxicity if any ≥ 6 weeks after the last dose of ^{131}I-MX35 F(ab')$_2$.

12.2 Patients will not be retreated with ^{131}I-MX35 F(ab')$_2$.

13 CRITERIA FOR THERAPEUTIC RESPONSE

Therapeutic responses are not the end point in this Phase I study. However, we will evaluate for response during the dose escalation portion of the study. Further, as all patients will have ≤ 0.5 cm diameter disease, conventional imaging studies will have limited value in the assessment of extent of disease and/or response. Serum CA 125 measurements may be used to monitor disease status in patients with an initial elevated value.

13.1 Patients WITHOUT bidimensionally measurable disease are evaluable by CA-125. Criteria for evaluating disease status with CA-125 levels are drawn from "Tumour markers", by G.J.S Rustin, M.E.L. van der Burg, and J.S. Berek.

13.1.1 Complete Response (CR): Normalization of the CA-125 for 3 successive evaluations, two weeks apart.

13.1.2 Partial Response (PR): A 50% fall in CA-125 after 2 samples confirmed by a fourth sample, two weeks apart; or a serial fall of 50% over three samples, two weeks apart; or a serial fall over three samples to less than 25% of the first sample.

13.1.3 Stabilization (STAB): Patients who do not meet the criteria for PR or PROG for at least 90 days will be listed in a stable disease category.

13.1.4 Progression (PROG): A 25% rise in CA-125 after 2 samples confirmed by a fourth sample, two weeks apart: or a serial rise of 50% over three samples, two weeks apart: or elevation above 100 μ/ml for over 2 months.

13.1.5 Performance Status: A Karnofsky performance status score will be assigned when evaluating patient. This will allow for changes in CA-125 to be correlated with changes in performance status.

13.2 Duration of response:

Non-measurable patients: Time from which the CA-125 is first noted to have decreased by ≥ 50% for three successive evaluations until a greater than 50% increase from the nadir value is documented on three

successive determinations.

13.3 Patients without evaluable disease will be assessed for toxicity alone.

14 CONSENT PROCEDURES

All patients will be required to sign a statement of informed consent.

15 STATISTICAL CONSIDERATIONS

This is a Phase I trial design, aimed to find the MTD of ^{131}I-MX35 F(ab')$_2$ monoclonal antibody. The dose escalation scheme is standard. At any dose level an initial 3 patients will be treated. If no Grade 3 or 4 toxicity is observed, dose escalation will occur. If 1 or 2 toxicities $\geq$ Grade 3 are observed, 3 more patients will be added at the same level. Dose will be escalated if only 1 of the 6 treated has $\geq$ Grade 3 toxicity. The MTD is the maximum dose at which at most 1 out of three, if only three were treated at that level, or 2 out of 6 show toxicity. The following gives the probability of escalation for a given true probability of toxicity.

Probability of Toxicity	10%	20%	30%	50%	75%
Probability of Escalation	91%	71%	49%	17%	1.8%

Since each dose level takes a maximum of 6 patients, assuming 4 dose levels, this trial would need a minimum of 3 patients and a maximum of 24 patients.

BIBLIOGRAPHY

1. Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics, 1996. CA *Cancer J Clin* 46:5-27, 1996.
2. NIH Consensus Development panel on Ovarian Cancer. Ovarian Cancer, Screening, Treatment, and Follow-up. *JAMA* 273:6:491-97, 1995
3. Vergote IB, Bormer OP, Abeler VM. Evaluation of CA-125 levels in the monitoring of ovarian cancer. *Am J Obstet Gynecol* 157:88-92, 1987.
4. Alvarez RD, To A, Boots LR, et al. CA-125 as a serum marker for poor prognosis in ovarian malignancies. *Gynecol Oncol* 26:284-89, 1987.
5. Rustin GJS, Nelstrope A, Stilwell MA, et al. Savings obtained by CA-125 measurements during therapy for ovarian carcinoma. *Eur J Cancer* 28:79-82, 1992
6. Epenetos AA, Nimmon CC, Arklie J. Antibody-guided irradiation of malignant ascites in ovarian cancer: a new therapeutic method possessing specificity against cancer cells. *J Obstet Gyn*; 68:71-74, 1986.
7. Epenetos AA, Munro AJ, Stewart S et al. Antibody-guided irradiation of advanced ovarian cancer with intraperitoneally administered radiolabeled monoclonal antibodies. *J Clin Oncol*; 5:1890-1899, 1987.
8. Stewart JSW, Hird V, Snook D, et al. Intraperitoneal Yttrium-90-labeled monoclonal antibody in ovarian cancer. *J Clin Oncol*; 8:1941-1950, 1990.
9. Muto MG, Finkler NJ, Kassis AI, et al. Intraperitoneal radioimmunotherapy of refractory ovarian carcinoma utilizing iodine-131 labeled monoclonal antibody OC125. *Gyn Oncol*; 45:265-272, 1992.
10. Crippa F. Radioimmunotherapy of ovarian cancer. *Int J Biol Markers*; 8:187-191, 1993.
11. Crippa F. Radioimmunotherapy of ovarian cancer. *Int J Biol Markers*; 8:192-197, 1993.
12. Colcher D, Esteban J, Carrasquillo, JA et al. Complementation of intracavitary and intravenous administration of a monoclonal antibody (B72.3) in patients with carcinoma. *Cancer Res* 4218-224, 1987.
13. Larson SM, Carrasquillo JA, Colcher DC et al. Estimates of radiation absorbed dose for intraperitoneally administered iodine-131 radiolabeled B72.3 monoclonal antibody in patients with peritoneal carcinomatosis. *J Nucl Med*; 32: 1661-1667, 1991.
14. Mattes MJ, Look K, Furukawa K, et al. Mouse monoclonal antibodies to human epithelial differentiation antigens expressed on the surface of ovarian carcinoma ascites cells. Cancer Res 1987; 6741-50.
15. Rubin S, Kostakoglu L, Federici M, et al. Clinical trial of pharmacokinetics, biodistribution, and intraoperative radioimmunodetection of radiolabeled monoclonal antibody (MAb) MX35 in epithelial ovarian cancer. Gynecol Oncol (abstr) in press, 1995.

Appendix A.

Observation	Pre Study[1]	^{131}I-MX35 $F(ab')_2$ ip on Day 1 During Therapy - week 1	2	3	4	5	6	7	Monthly until EOS*
Pathology Review	X								
History	X**				X				
Physical Exam	X**				X				
Weight and Height	X**				X				
Performance Status	X**				X				
CBC, Diff, Platelet	X**		X	X	X	X	X	X	
PT, PTT	X**				X				
Chemistry Survey[2]	X**				X				
CA-125	X**		X		X		X		X
EKG	X**								
HAMA	X**				X				X
I-131/Tc-99m serum estimation[3]		Pre- and 15 minutes, 1h and 2h post- infusion of ^{131}I-MX35 $F(ab')_2$; then daily for a week; 2 weeks.							

I-131/Tc-99m ip estimation[3] 15 minutes, 1h and 2h post- infusion

1: Within 2 weeks prior to starting ^{131}I-MX35 $F(ab')_2$.

*: EOS: End of study. Refers to time to disease progression, or entry into another therapeutic protocol.

**: Prior to retreatment also.

2: Includes SGOT, LDH, Alk Pho., bilirubin and creatinine.

3: 2 ml pediatric red top

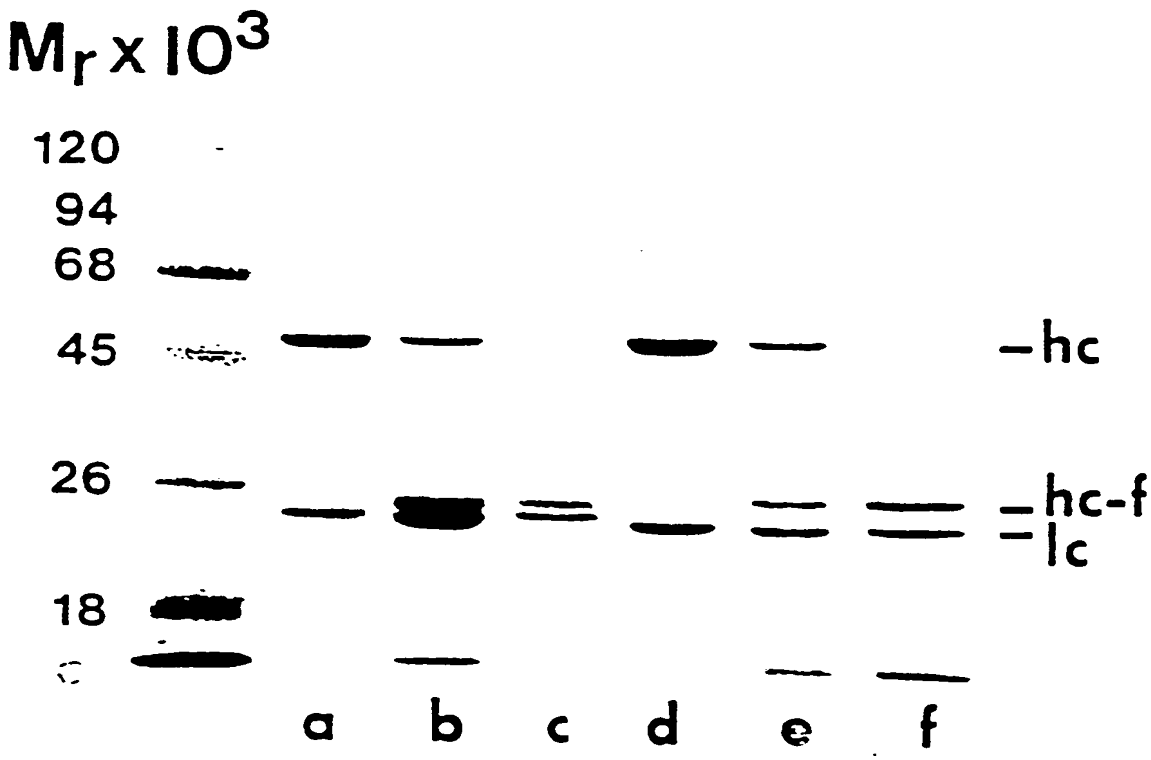


FIGURE 1

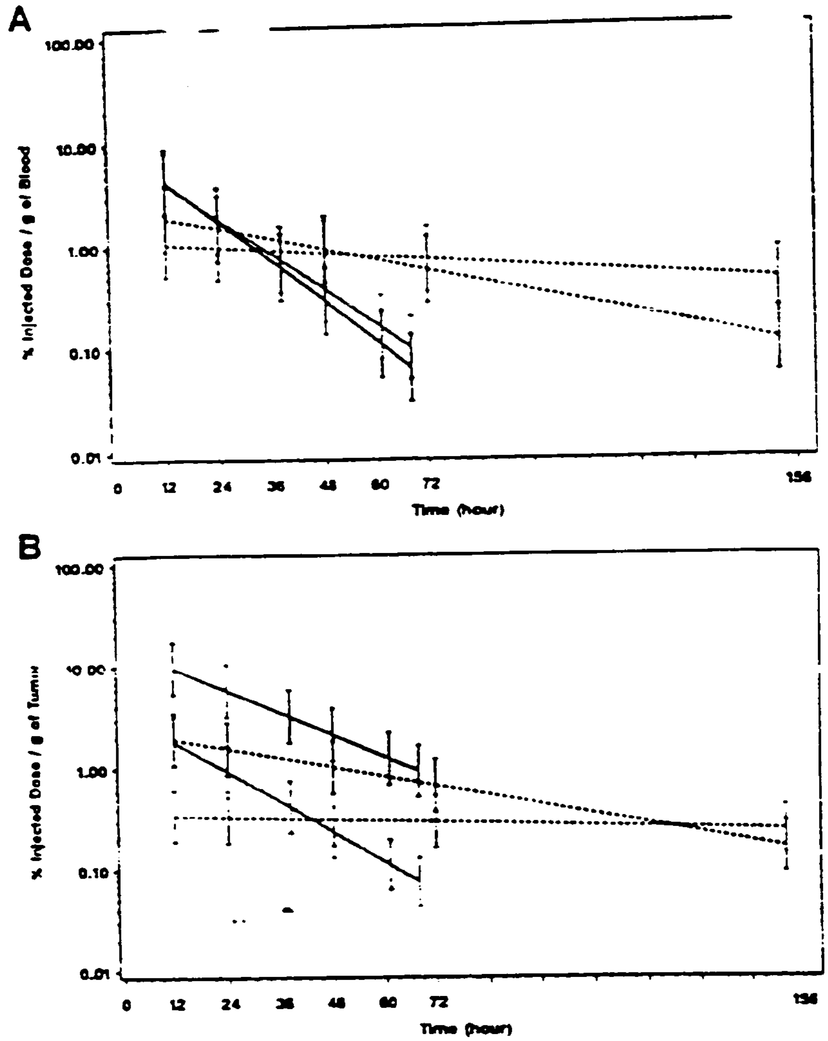

A
100.00
10.00
1.00
0.10
0.01
% Injected Dose / g of Blood
0 12 24 36 48 60 72 156
Time (hour)
B
100.00
10.00
1.00
0.10
0.01
% Injected Dose / g of Tumor
0 12 24 36 48 60 72 156
Time (hour)

FIGURE 2

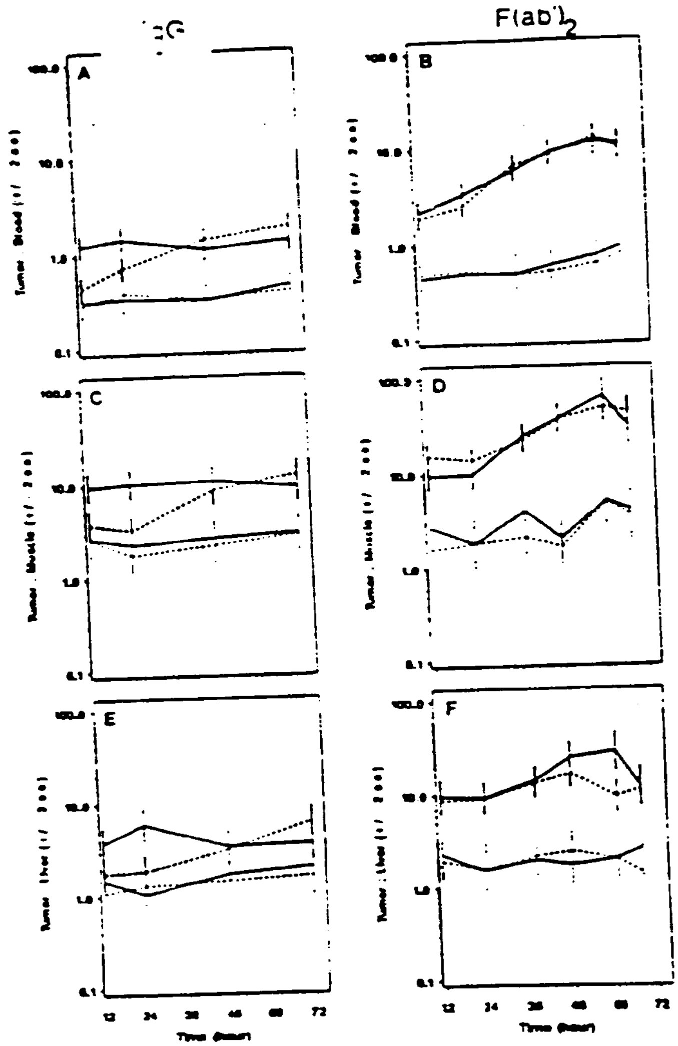


FIGURE 3

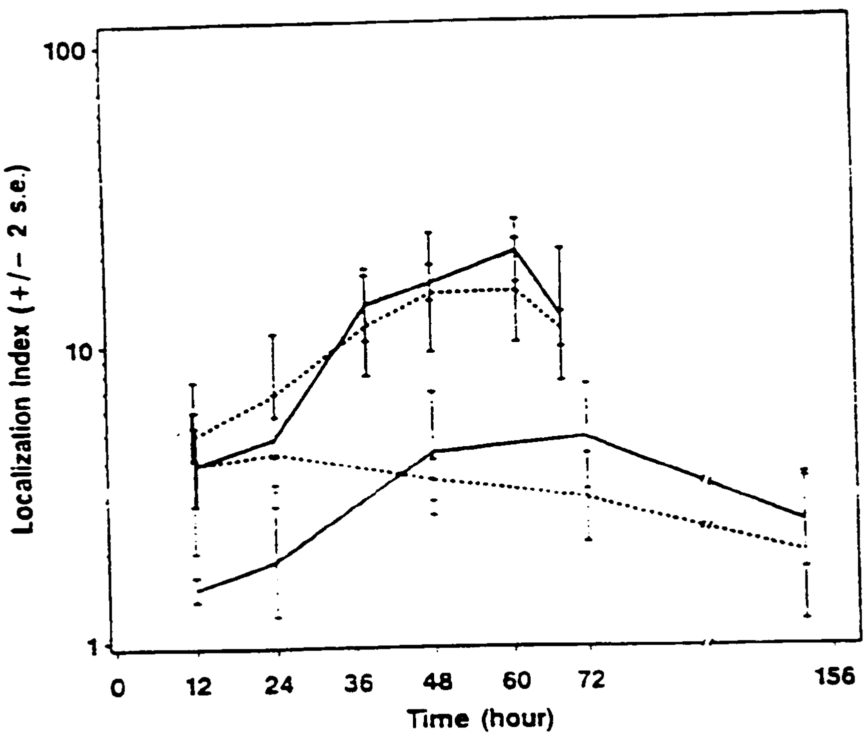

100
10
1
Localization Index (+/- 2 s.e.)
0
12
24
36
48
60
72
156
Time (hour)

FIGURE 4

INTRODUCTION

In the treatment of advanced epithelial ovarian cancer, the combined effect of surgery and chemotherapy has resulted in a complete response rate of 45% as confirmed by reassessment surgery [1]. However, the risk of recurrence remains high in patients with advanced disease (stages III and IV) with 50% recurring within a median of 14 months after negative second-look laparotomy [2]. Patients with residual disease detected at second-look surgery or recurrent disease after completion of initial chemotherapy have a poor prognosis and few, if any, are cured by currently available salvage therapy. The potential of radiolabeled monoclonal antibodies (mAb) for the detection and quantitation of metastatic spread offers significant benefits for the subsequent management of these patients, as well as the possibility to actually treat micrometastatic disease with antibody carrying the appropriate therapeutic radionuclide, toxin or drug.

The application of radiolabeled antibodies for both radioimmunodiagnosis and treatment of ovarian carcinoma has been ongoing for more than 10 years [3-10]. Epenetos et al. [4] and Pateisky et al. [5] used ^{131}I and ^{123}I labeled antibodies (HMFG1 and HMFG2) against peptide epitopes of human milk fat globulin. Using gamma camera scintigraphy, they successfully demonstrated that >75% of patients having metastatic spread into the peritoneum imaged positively. Negative scans were attributed to the absence of disease or the presence of unresolvable microscopic foci only. The lack of solid tumor nodules >1.5 cm in diameter would render insufficient image contrast to enable specific antibody binding to be detected against a non-specific background [11]. Neither gamma camera imaging nor hand held surgical radioactivity probes [8, 12] exhibited the sensitivity required to detect micrometastatic disease (<1 cm in diameter), due to insufficient contrast (rarely >10:1) of radionuclide activity accumulation within the tumor relative to the peri-tumor region [11].

Micrometastatic disease may, therefore, remain undetected by conventional nuclear medicine procedures. Moreover, in biodistribution studies using biopsied specimens, the presentation of radiolabeled antibody uptake and dosimetry as an activity per unit gram of tissue can be in significant error. This is because of the small size of the biopsy and the presence of only clusters of tumor cells within a large region of stromal tissue, endothelium and hematopoietic cells. Thus, including non-tumor cells in the activity per unit gram calculations can greatly dilute the tumor specific activity.

In order to explore ways around this problem, we have examined the use of storage phosphor screen technology to determine the distribution of radioactivity in surgical specimens obtained from an ongoing antibody imaging trial on the use of the radiolabeled murine monoclonal antibody (mAb) MX35 F(ab')2 fragment in patients with ovarian carcinoma having minimal residual disease. Digital images from scanned storage phosphor screens were compared with autoradiographic images obtained using film techniques and MX35 antigen localization determined by indirect immunohistochemistry in order to confirm the specific uptake of radiolabeled-mAb MX35 F(ab')2 to tumor cell foci. The data from phosphor digital images were used to evaluate the radionuclide distribution and to estimate accumulation in micrometastatic tumors, adjacent non-tumor tissue and other normal tissue samples. These estimates of tumor specific activity and radiation dose measurements were compared with traditional estimates of the percentage injected dose per gram of tissue (%ID/g) by well scintillation counting.

MATERIAL AND METHODS

Patient Selection

Patients in this study had undergone prior surgery for epithelial ovarian cancer and had completed a prescribed course of platinum-based chemotherapy. Eligibility criteria included: known or suspected carcinoma of the ovary, a Karnofsky performance status greater than 60, no prior administration of murine mAb or fragment, and/or a negative human anti-murine antibody (HAMA) titer. Informed consent was obtained from all patients before participation in the study; the study and consent forms were approved by the Institutional Review Board of Memorial Hospital (IRB 94-13). Prior to participating in this trial, either paraffin-embedded tumor specimens or fresh, frozen tumor specimens from an earlier surgery were examined by immunohistochemistry for expression of the MX35 antigen on at least 75% of the carcinoma cells. The tissue specimens from six patients, who have participated in the ongoing monoclonal antibody imaging trial prior to a second-look surgery, are summarized in Table 1.

Preparation of Radiolabeled Monoclonal Antibody MX35 F(ab')2

Monoclonal antibody MX35, a murine IgG1, was generated from the hybridoma fusion of NS-1 murine myeloma cells with splenocytes from a mouse immunized with 4 fresh ovarian carcinoma specimens [13] and purified as described previously [14]. For fragmentation, purified mAb MX35 IgG (2 mg in 400 μl) was dialysed overnight in 0.1 M citric acid buffer, pH 4.5. Pepsin (25 μl of 1.5 mg/ml; Sigma Chemical Co., St Louis, MO) was then added to the antibody and digested for 3 hours at 37°C with agitation. F(ab')2 fragments were isolated using an Avid Chrom F(ab')2 Kit (UniSyn Technologies Inc., Tustin, CA). High yield binding buffer (250 μl) containing 30 μl of anti-pepsin was added to the antibody-pepsin combination. The entire sample was diluted with 220 μl of high yield binding buffer and centrifuged to 2000 rpm for 15 minutes and the supernatant was placed on a protein-A-Avid Chrom cartridge. The unadsorbed fraction was concentrated using a Centricon 30 unit (Amicon, Beverly, MA) at 1075 g at 4°C. The final antibody concentration obtained was 9.3 to 13.6 mg/ml. The identity of the fragments was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions; staining of the gel with Coomassie blue, revealed bands of 23 kD (light chains) and 25 kD (cleaved heavy chains).

The mAb MX35 F(ab')2 fragments were radiolabeled with iodine radionuclides using the chloramine-T method as follows: Two milligrams of antibody fragments were added to 0.5 ml of 0.10 M phosphate buffer, pH 7.4. To the radionuclides, ^{131}I

and ^{125}I, was added 100 µl of phosphate buffer and this solution was added to the antibody fragment solution. Chloramine-T (2 µg/ml) in phosphate buffer was added and after 2 minutes the reaction was quenched by addition of 50 µl sodium metabisulfate (10 µg/ml). The protein was separated by passage through a Biogel P6 column (10 ml; BioRad, XXX) using 1% human serum albumin in 0.15 M NaCl as eluant. Terminal sterization was achieved by filtering through a 0.22 micron filter. Immunoreactivity of the labeled product was determined by sequential absorptions with an antigen-expressing cell line (OVCAR-3). Between 50 to 65 percent of the radioactivity was adsorbed using the method described by Mattes et al. [15]. Percent labeled protein was determined by thin layer chromatography and incorporation of radiolabeled iodine into protein were >95%. All procedures were performed aseptically with pyrogen-free material.

Administration of Radiolabeled Monoclonal Antibody MX35 F(ab')2

Beginning at least 24 hours prior to antibody administration and continuing to the time of surgery, patients were treated orally with 10 drops of a saturated solution of potassium iodide three times daily. $^{131}I/^{125}I$-labeled mAb MX35 F(ab')2 was administered by intravenous (IV) route in a 0.9% sodium chloride solution containing 5% human serum albumin (total volume 100 ml) through a 0.2 micron Millex G-V filter (Millipore, Bedford, MA) over a period of 1 hour. Radiolabeled mAb MX35 F(ab')2 was administrated by intraperitoneal (IP) route as follows: 500 ml of 0.9% sodium chloride was delivered using a catheter or a pre-existing IP port into the peritoneal cavity to facilitate antibody distribution; 100 ml of radiolabeled antibody was added in the same solution as the IV route; and an additional 500 ml of 0.9% sodium chloride was delivered. Five patients were entered at the 2 mg antibody dose labeled with both ^{131}I and ^{125}I. Three patients were injected by IV route and 2 patients by IP route. One patient was entered at the 10 mg antibody dose [2 mg of radiolabeled antibody plus 8 mg of unlabeled mAb MX35 F(ab')2] and injected by IV route.

Blood Samples and Tissue Biopsies

Blood samples were drawn pre-infusion of radiolabeled mAb, post-infusion (1 to 4 hours), immediately before surgery in order to compare radiolabeled antibody levels in the blood with those in the biopsied material, and 4 to 7 days post-surgery. Whole blood was centrifuged at < 2000 rpm for 10 minutes, serum was aspirated, and one ml of pre-surgery serum was weighed and counted in a Packard Cobra well scintillation counter (Packard Instrument Company, Douners Grove, IL).

Multiple biopsied specimens including adjacent normal tissue (fat, muscle

and/or peritoneal wall) were retrieved from 6 patients during second-look surgery as summarized in **Table 1**. Fresh surgical biopsies were divided as follows: One half of each specimen was paraffin-embedded and used for routine histologic evaluation in our Department of Pathology. The other half of each biopsy was weighed and counted in a Packard Cobra well scintillation counter and then snap frozen in liquid nitrogen, embedded in Optimal Cutting Temperature (OCT) compound (Miles Laboratory Inc., Elkhart, IN) and stored at -80°C. A proportion of the frozen surgical biopsies from each case were cut using a motorized cryostat (Bright Instrument Co., Huntingdon, England, UK) and air dried onto microscope slides. Adjacent tissue sections (6 micron thickness) from each biopsy were then analyzed for MX35 antigen localization using an indirect immunoperoxidase procedure and for radionuclide distribution by autoradiography using film and storage phosphor screens.

The number of counts per minute (cpm) were obtained in two windows centered at 25 keV for ^{125}I and 364 keV for ^{131}I. The cpm was converted into activity by measuring ^{125}I and ^{131}I standards alongside the tissue specimens. The %ID/g for each radionuclide was determined for the blood and each tissue biopsy by dividing the specific activity (i.e., $\mu Ci/g$) by the activity administered to the patient and multiplying by 100.

Immunohistochemical Analysis and MX35 Antigen Localization

Frozen tissue sections were fixed with cold acetone and analyzed for reactivity with mAb MX35 as described previously **[16]**. Antibody staining patterns were scored in a semiquantitative fashion. Specimens were classified as showing strong (+++) antigen expression when 75% or more of the tumor cells were stained, heterogeneous (+ to ++) expression when 10 - 75% of the tumor cells stained, and no expression when negative or less than 10% of the tumor stained.

Autoradiography using Film for Distribution of Radionuclide Activity

Autoradiography was performed using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). Tissue sections were covered with Saran wrap and overlaid with film alone or with an enhancer screen for exposure durations of between 1 day to 14 days. Films were developed in a Kodak RP X-OMAT processor (Eastman Kodak). Film images were digitized using the Nikon CoolScan (Nikon, COMPANY) and compared to the digitized 35 mm Ektachrome film images of adjacent tissue sections immunostained with mAb MX35. Co-registration of the serial sections permitted visualization of the radiolabeled antibody activity over regions of tumor cells and non-tumor tissue.

Autoradiography using Storage Phosphor Screens for Distribution of Radionuclide Activity

For the analysis of storage phosphor screen images either a Model GS-250 or Model GS-350 Molecular Imager™ system (Bio-Rad Laboratories Inc., Genetics Systems Division, Hercules, CA) connected to a Macintosh XXX computer system (Apple Computers Inc., Cupertino, CA) was used. The storage phosphor screen (type BI) is fabricated from strontium sulfide commingled with elemental cerium and samarium (SrS; Ce, Sm) [17]. The interaction of ionizing radiation with the storage phosphor screen excites electrons into the conduction band from which they fall into electron traps. Quantitation of the number of filled traps is proportional to the amount of energy deposited in the screen. The detector signal is read out using an externally applied scanning laser (pulsed infrared diode at 910 nm) which scans the screen releasing the electrons from their traps, pixel by pixel. The process of electron de-excitation results in the emission of fluorescent photons, which are collected in a fiber optic pipe and counted using a photomultiplier tube. The resultant signal is processed using an analog to digital converter which provides the final 10 to 16 Mbyte image at 16 bit per pixel. The phosphor image data were analyzed on a Power Macintosh 7100/66 (Apple Computers Inc.) using Molecular Analyst™/Macintosh (Version 2.0) data analysis software (Bio-Rad Laboratories Inc.) and the public domain NIH Image (Version 1.55) program (written by Wayne Rasband, U.S. National Institutes of Health) which is available from the Internet.

Tissue sections were covered with Saran wrap and then clamped against a storage phosphor screen in a light-tight cassette for a preliminary exposure duration of 24 hours and a second exposure duration of 4 to 16 days. The storage phosphor screen was erased after image read-out by exposure to infrared light for 15 minutes, and erased a second time immediately before re-exposure to the samples.

Calibration of the Storage Phosphor Screen using Radionuclide Standards

A storage phosphor screen image consists of a 2-dimensional array of intensities. The conversion of these intensities into specific activity units requires calibration of the storage phosphor screen response. The screen response was analyzed with three sets of radiolabeled standard sources. Strips containing graded standards of ^{14}C (RPA.504 and RPA.511) and ^{125}I (RPA.523) for autoradiographic calibration were purchased (DuPont, New England Nuclear COMPANY). The commerical ^{125}I standards embedded in polymer were quoted at 20 μm tissue equivalent thickness. A set of ^{131}I standards were made by dilution of a stock solution containing a known activity of ^{131}I as follows: Eosin Y (1% alcoholic

solution; Polysciences, Inc. Warrington, PA) was added to the radioactive solution, which was then mixed with the OCT compound until a uniform coloration was achieved. The samples were weighed and counted and the relative specific activities of the two blocks determined to be 0.536 µCi/g and 4.680 µCi/g respectively. The two ^{131}I standards were sectioned at 6 mm thickness and dried onto microscope slides in the same way as the tissue sections prior to exposure to the screen for 24 hours. The response of the phosphor screen was determined to be 1814, 4740, and 830 counts per day (cpd) per pixel for ^{131}I, ^{125}I and ^{14}C, respectively for a source of 1 µCi/g specific activity. The higher sensitivity of the storage phosphor screen means that exposure times are typically between 5 to 10 times shorter than film for the same image quality [18]. The ^{14}C standards were placed alongside the iodine standards to evaluate the constancy of the storage phosphor screen over a long period.

The storage phosphor screen exhibits a slow signal fade during the signal acquisition time. Thus, for each sample exposure time, it was required to convolute the signal accumulation with the signal fade. The fade characteristics were determined by repeatedly exposing the screen to the standards for the same one hour duration, and varying the interval before reading the screen, from immediate up to 14 days. The correction factor to account for signal fade (F) is given by the convolution integral $\int_0^{to} e^{-\lambda_F \tau} . e^{-\lambda_P (t - \tau)} d\tau$, where λ_F is the rate of signal loss attributed to fade obtained by fitting the signal counts per day versus the time interval from exposure to read-out and λ_P is the physical decay constant for the respective radionuclide. Solving the integral, one obtains

$$F = e^{-\lambda_P t_o} [1 - e^{-(\lambda_F - \lambda_P) t_o}] / (\lambda_F - \lambda_P).$$

The rate constant λ_F for phosphor screen fade was found to be 0.0967 per day. Inserting the decay constant λ_P for ^{131}I, the above equation becomes

$$F = e^{-0.0862\, t_o} [1 - e^{-0.0105\, t_o}] / 0.0105,$$

where t_o is the time in days the phosphor screen is exposed to an ^{131}I-labeled specimen.

Estimation of Dosimetry using Storage Phosphor Screens

The radiation dose is directly proportional to the cumulative specific activity of the radiolabeled antibody in the tumor. The dosimetry estimates presented in this paper were derived from the storage phosphor screen images. The film autoradiographs were used for high resolution visualization only. We assumed a biological half-life (T_b) in the tumor of 15.5 hours for both IV and IP routes of

injection [19]. This estimate was based upon measurements with the same radiolabeled ^{131}I-labeled mAb MX35 F(ab')2 in a murine xenograft tumor model with OVCAR-3 human ovarian cancer cell line. This half-life is reasonable for F(ab')2 antibodies used in the treatment of ovarian carcinoma as evidenced by biological half-lives reported in patient trials after intraperitoneal injection with other radiolabeled mAbs, e.g., T_b = 21 hours for ^{111}In-mAb OC125 [20] and T_b =14 hours for ^{186}Re-mAb NR-CO-02 [21]. The physical half-life (T_p) is 8.04 days for ^{131}I and 60 days for ^{125}I. The biological half-life results in an effective half-life (T_e) where $T_e = T_b \cdot T_p/(T_b + T_p)$ = 14.3 hours for ^{131}I and 15.3 hours for ^{125}I. In this clinical trial the principal radionuclide dose contribution came from either ^{125}I (Cases 1 and 2) or ^{131}I (Cases 3 - 6) and the calculations which follow focused on the dosimetry for the appropriate radiolabeled mAb MX35 F(ab')2.

The "peak" specific activity in a biopsy specimen is back extrapolated from measurements of the whole biopsy by well scintillation counting, at 1, 4, or 5 days post infusion of the radiolabeled antibody, using the assumption of a monoexponential clearance rate which equals $C_o\, e^{-\lambda T_e}$, where λ = 0.693/14.3 hours for ^{131}I and λ = 0.693/15.3 hours for ^{125}I and C_o is the specific activity in µCi/g extrapolated back to time t=0.

The radiation absorbed dose resulting from the specific activity is given by the integral of the specific activity $C_o \int e^{-\lambda T_e}$ over time multiplied by 2.13 $\Sigma n_i E_i \cdot \phi_i$. This equation consists of the product between the total energy ($\Sigma n_i E_i$) released by the radionuclide emission, the fraction of the emission energy absorbed within the tumor (ϕ), and 2.13 which is a units conversion factor. For ^{131}I, the sum of the beta ray energy emitted per decay ($\Sigma n_i E_i$ = 0.187 MeV) multiplied by 2.13 equals 0.398 g-cGy/µCi-hr. For non-penetrating radiations, such as beta particles, it is recommended by the MIRD committee to use unity for the absorbed fraction (ϕ) [22]. However, for a micrometastatic deposit, containing less than a gram of tumor cells, the value of ϕ is less than one. The absorbed fractions for several radionuclides including ^{131}I are published by Humm [23] and Goddu, Rao and Howells [24]. For a 100µm micrometastatic lesion, ϕ is equal to 0.17, i.e., 17% of the energy emitted within the lesion is deposited locally.

RESULTS

From an ongoing study on the localization of radiolabeled mAb MX35 F(ab')2 in patients with ovarian carcinoma, biopsied specimens were used to compare the distribution of radionuclide as determined by well scintillation counting of whole tissue specimens with storage phosphor screen autoradiography of tissue sections. These specimens were taken during second-look surgery after antibody administration, 1 day to 5 days earlier. Biopsied specimens from 6 patients were analyzed (**Table 1**). All tumors were shown to express MX35 antigen as determined by immunohistochemical analysis. In total, 19 normal tissue biopsies and 16 biopsies containing tumor cell foci were studied in the laboratory. Tissue biopsies were counted for ^{131}I and ^{125}I activity in a well scintillation counter and cryostat sections, from these biopsies, were exposed to film and storage phosphor screens as outlined in the Materials and Methods section.

Determination of Specific Activity of Radiolabeled Antibody in Whole Tissue Biopsies using Well Scintillation Counting

The %ID/g was calculated for the whole biopsy specimens and blood sample for each case (**Table 2**). The %ID/g for biopsies with tumor ranged from 0.5 to 8.7×10^{-3} (for ^{131}I calculations) and from 0.3 to 6.4×10^{-3} (for ^{125}I calculations) for samples analyzed between 4 to 5 days post surgery. The %ID/g for a single tumor sample studied one day post surgery was 22×10^{-3}. The tumor:blood ratios ranged from 0.2:1 to 2.8:1 in the patients receiving antibody by the IV route and 3.7:1 to 5.6:1 by the IP route. The tumor:normal tissue (fat) ratios ranged from 0.9:1 to 39:1. The tumor:normal tissue ratios were greater in the two patients (cases 4 and 5) receiving antibody via the IP route. The percentage of tumor cells within the biopsy specimens was variable, ranging from <10 % to >75% of a sample (**Table 1**). In 2 of 6 cases (cases 2 and 4), greater than 50% of the biopsy consisted of tumor foci and in these cases the tumor:normal tissue ratios were significant (18:1). In 3 of 6 cases, less than 20% of the biopsy consisted of tumor foci and the tumor:normal tissue ratios were in the range between 0.9:1 to 8.9:1. One specimen (case 5) with <10% tumor cells in the biopsy had the highest tumor:normal tissue ratio of 36:1.

Immunohistochemical Delineation of Areas of Tumor Cells in Tissue Sections and Comparison with Film and Storage Phosphor Screen Autoradiography.

The autoradiographs from film and storage phosphor screens provide an image of the distribution of radionuclide *devoid of its relation* to the histology of the tissue section. Co-registration of phosphor images and/or digitized film images with the digitized Ektachrome images of tissue sections, stained by immunoperoxidase

using mAb MX35 for antigen localization, allowed visual assessment of the efficacy of radiolabeled mAb targeting. Film images of tumor and normal tissue sections were available for 6 cases. Clusters of tumor cells were clearly detected by film visualization in 5 of 6 cases; in the other case single tumor cells and small clusters of <10 tumor cells were weakly detected by film (case 3). Phosphor screen images were analyzed for tumor and normal tissue sections in 5 cases; single tumor cells and small tumor cell islets did not image clearly in case 3.

Analysis of adjacent tissue sections confirmed the coincidence of the radiolabeled mAb MX35 F(ab')2 to regions of MX35-positive tumor cells. **Figures** 5–7 illustrate the results from three cases comparing film with storage phosphor screen images. **Figure** 5 illustrates a small laparoscopy specimen (case 2) with >80% of the specimen having strong MX35 antigen expression on the serous type ovarian carcinoma cells at high power (Figure 5a). Co-registration of the tissue section immunostained for antigen localization (Figure 5b) with both the film autoradiographic image (Figures 5c) and the digital phosphor screen image (Figure 5d) shows variable intensity in the areas of radiolabeled mAb accumulation. **Figure** 6 illustrates a para aortic lymph node specimen (case 5) with a micrometastatic tumor cell cluster from a poorly differentiated ovarian carcinoma detected by immunostaining for antigen localization at high power (Figure 6a) and low power (Figure 6b). The film image has saturated at the 7 day exposure (Figure6c). The phosphor screen image has a greater dynamic range and therefore does not saturate (Figure 6d). The one day film and phosphor screen images showed specific radionuclide localization to the same clusters of tumor cells as detected by immunostaining (data not shown). **Figure** 7 illustrates an endometrioid carcinoma of the peritoneum (case 6). A micrometastatic tumor cell cluster was found near the surface of the ovary (upper panel) and multiple clusters of tumor cells were detected adjacent to the Fallopian tube epithelium (lower panel). The small lesion in the ovary (Figures 7a and 7b) is specifically targeted by radiolabelled mAb MX35 F(ab')2 as seen in the autoradiographic film and phosphor screen images (Figures 7c and7d .). Note that the Fallopian tube epithelium expresses MX35 antigen along the apical surface (Figures 7e and 7f) but the radiolabeled antibody preferentially localizes to the tumor cell clusters (Figures 7g, and 7h).

Determination of Specific Activity ot Radiolabeled Antibody Within Tissue Specimens using Storage Phosphor Screens

Line profiles were drawn so as to traverse regions of micrometastatic tumor cell foci which impregnate the regions of normal stromal tissue. Ratios of the "peak"

response (converted to cpm or cpd) overlying the MX35-positive tumor deposits relative to the normal stromal tissue background can differ significantly, as illustrated in **Figure** 8 from case 4. By correcting the signal accumulation for physical decay and signal fade the "relative" specific activity for regions of interest within the phosphor screen image are obtained. These estimates render an assessment of the local relative specific activity of the radiolabeled antibody in the tumor and adjacent normal tissue within a tissue section. Estimates of the tumor activity was compared with measurements of the average activity per gram for the whole biopsied specimen from a well scintillation counter (Table 3).

The phosphor screen images were analyzed retrospectively for all patients, after ascertaining the phosphor screen response, i.e., the counts per day per pixel per unit specific activity, as a function of exposure duration from standards. ^{131}I, ^{125}I, and ^{14}C standards were exposed alongside sections of both normal and tumor tissues for patients 5 and 6. This allowed the unknown activity distribution in the biopsied specimen to be determined by direct scaling from the known activity of the standards and the ratio of the radionuclide response. The validity of our approach to patients 1 to 4, who were studied prior to the simultaneous exposure to standards, was verified by estimating the specific activity for patients 5 and 6 using both methods. The ratio between the values obtained by the new method involving simultaneous exposure to standards, to the previous method, by applying the known response and fade characteristics of the phosphor screen, was 0.86 and 0.95 for cases 5 and 6, respectively.

Estimation of Dosimetry Within Tissue Specimens using Storage Phosphor Screens

Tables 2 and 3 summarize the dosimetry results for mAb MX35 F(ab')2 in tumor biopsy samples and normal tissues. The %ID/g and range of tumor:normal tissue ratios determined by a calibrated well scintillation counter on the day of biopsy, are presented in Table 2. In Table 3, the %ID/g from Table 2 are compared with estimates of local activity data derived from storage phosphor screens. Assuming an effective half life of 14.3 hours for ^{131}I and 15.3 hours for ^{125}I, the %ID/g of tissue was back extrapolated to zero time and the cumulative specific activity calculated, i.e., the area under the curve using a monoexponential clearance.

The radiation dose estimates derived from the well scintillation counter measurements for the 4 patients showing good radionuclide localization to MX35-positive tumor cells were 70.1, 10.9, 106.3 and 7.7 cGy/mCi for patients 2, 4, 5 and 6, respectively. These estimates assume a uniform distribution of radionuclide activity within the tumor and do not take into account the complex microscopic distribution of the activity at the cellular level. The dose deposited in small microscopic foci of

disease would be substantially reduced below estimates based upon the assumption of charged particle equilibrium [23] as a consequence of the small fraction of energy emanating from the high energy β-particles of ^{131}I, which is deposited locally. The fraction of ^{131}I β-ray energy locally absorbed within a 100μm diameter lesion is 0.17 [24]. This would reduce the dose estimates to only 8.9, 1.85, 18.07 and 1.31 cGy/mCi of ^{131}I injected for patients 2, 4, 5 and 6, respectively. However, the storage phosphor screen autoradiographs show that the activity is not uniformly distributed through the biopsied specimens, but that "hot spots" of activity accumulate at the microscopic tumor lesions. From analysis of the counts per day overlying the tumor regions from the phosphor image data, we observed that specific activity was greater in tumor by a factor of between 4 to 12 times that predicted/estimated from well counter measurement. This is because the well counter averages the activity per gram over both the tumor and stromal cells of the biopsied specimen. The %ID/g determined by the well counter and storage phosphor screen techniques are summarized in **Table 3** alongside the dose estimates to the micrometastatic deposits by both methods. The absorbed doses determined by the storage phosphor screen are mostly greater than those predicted from the well counter method, even after consideration of the reduced absorbed fraction in a microscopic lesion. The phosphor screen method provides information about the activity distribution at a single time point from individual tissue sections and allows estimates to be made of the doses, that can be achieved within microscopic disease per mCi of ^{131}I-mAb MX35 F(ab')2 administered.

DISCUSSION

In this study, we evaluated the radiolabeled antibody uptake of the murine mAb MX35 F(ab')2 in biopsied samples from patients with epithelial ovarian cancer by well scintillation counting and by autoradiography using storage phosphor screens. Specific localization of mAb in tumor was demonstrated by co-registration of the immunohistochemical staining in areas of tumor cell clusters with autoradiographic film and phosphor screen images. In all specimens with micrometastatic spread, radiolabeled mAb uptake showed specific localization to the carcinoma cells (**Figures 5–7**). Factors, other than antigen distribution, are involved in the localization of radiolabeled antibody in tissues. In this study we noted that antibody localized to tumor cell foci but did not accumulate in the adjacent normal antigen-positive Fallopian tube epithelium (**Figure 7**) in the tissue sample. In this case accessibility of the antibody to the luminal side of the ducts may be limited.

The radiolabeled antibody uptake (%ID/g) determined by well scintillation counting (1 to 5 days post mAb infusion) ranged between 223.5 to 5.2×10^{-4} %ID/g of tissues for ^{131}I and 210.9 to 2.9×10^{-4} %ID/g for ^{125}I. There was a general relationship between the radiolabeled mAb uptake in the tumor biopsy and both the level and the intensity of the immunohistochemical expression of the MX35 antigen in the corresponding tumor tissue section. Specific localization of mAb in tumor was demonstrated by tumor:normal tissue (fat) ratios ranging from 0.9:1 to 35.9:1 for ^{131}I and from 0.9:1 to 39.0:1 for ^{125}I. Significantly higher tumor:normal tissue ratios were calculated for the two patients given radiolabeled antibody by the IP route (e.g., 17.7:1 and 35.9:1 for ^{131}I). These results can be compared to an earlier clinical trial in which mAb MX35 whole IgG was used [8]. In that study, tumor samples obtained at surgery (7 to 20 days post mAb infusion) showed a mAb accumulation of between 67.0 to 0.3×10^{-4} %ID/g of tissue and the tumor:normal tissue (fat) ratios ranged from 2.3:1 to 34.4:1. The tumor:normal tissue ratios were not significantly related to mAb dose, the level of immunohistochemical antigen expression or the interval between mAb infusion and surgery [8]. Also, in contrast to the present study, **tumor:serum** ratios rarely exceeded 1.0.

Our analysis provided a measure of the specific activity of biopsied specimens at one single time point; namely, at the time the biopsied specimen was removed and frozen. Because there was no means to determine the specific activity and microdistribution of the radiolabeled antibody before this time, we assumed an effective half-life for the ^{131}I-labeled mAb MX35 F(ab')2 clearance of 14.3 hours based on a parallel murine study performed in our laboratory [19]. Quantitative

autoradiography using film is complex with significant limitations, when the activity distribution is non-uniform, due to the limited linear response of film [28]. Therefore, we used storage phosphor screens which have a broader (>4 logs) linear response in this study. Measurements of the specific activity in the tumor, determined by the storage phosphor screen technique, are 4 to 12 times greater than those obtained by well scintillation counter estimates from the biopsied specimens (**Table 1**). These ratios do not directly translate to the absorbed dose estimates, which must further consider the size of the tumor cell clusters, and correct for the fraction of local energy absorption. Such corrections to absorbed dose are not possible when well counter methods are used since it is assumed that the radiolabel is uniformly distributed throughout the specimen. The storage phosphor screen approach can account for local energy depositions and thereby render accurate dose estimates with which to predict radiotoxicity. If dose limiting toxicity to the bone marrow is reached following a single administration of 100 mCi then the expected doses presented in **Table 3** may be multiplied by 100. Thus, doses to the micrometastatic deposits within patients 2, 4, 5, and 6 could be as large as 5200, 2660, 8140 and 1470 cGy, respectively. In vitro studies on the radiosensitivity of ovarian carcinoma cell lines, OVCAR-3 and OVCA-433, have demonstrated that a radiation dose of approximately 1100 cGy is required to reduce the fraction of cell survivors to 0.001, ie., 3 logs of cell kill [28]. Estimates of the localization of radiolabeled mAb MX35 F(ab')2 within micrometastatic lesions by phosphor screen techniques represent one of the most accurate assessments to date of the specific activity which can be targeted to microscopic disease.

To improve the radiation dose estimates presented here, it would be necessary to determine the specific activity of the radiolabeled antibody in the tumor at multiple time points. This information is not available for microscopic disease. Griffith et al. [25] proposed a method to implant thermoluminescent dosimeters, mounted at the tip of a catheter into tissue, in order to directly measure the radiation dose *in situ*. Yet, this method would not be readily applicable to microscopic disease, due to the uncertainty of the location of the tumor cells. A method using Positron Emission Tomography (PET) to assess quantitatively mAb localization *in situ* has been developed and tested with ^{124}I-labeled mAb 3F8 in a patient with glioma [26]. High-resolution PET has been used to localize human ovarian cancer in nude rats using ^{124}I-labeled mAb MX35 [27]. The high sensitivity of PET may allow the detection of microscopic disease. However, the resolution of current PET scanners in the abdomen is not better than 4 mm (General Electric Advance PET Scanner,

Milwaukee, WI).

When an antibody can be shown convincingly to localize to micrometastatic tumors, epithelial ovarian cancer affords an ideal opportunity to use the antibody or antibody conjugates for therapy. The specific targeting of mAb MX35 F(ab')2 to micrometastatic disease as shown in this study demonstrates the potential of this radiolabeled antibody conjugate for such a therapeutic trial. The ability to target minimum residual disease may be a significant rationale for treating patients with refractory or recurrent ovarian cancer with radioimmunotherapy. Small tumors may be more uniformly accessible to monoclonal antibody and require lower doses of radiation than bulky disease. Ovarian cancer often spreads superfically on the surface of the peritoneum where it forms small tumor foci within the peritoneal cavity. Extraperitoneal metastases, other than spread to local lymph nodes, are rare. Administration of antibody through an IP route, therefore, provides an optimal mode for the treatment of this disease. An additional advantage of IP administration is that, although small tumors may be under vascularized, they will still be accessible to radiolabeled antibody by diffusion from the peritoneal fluid.

This study has demonstrated that mAb MX35, in its F(ab')2 form, avidly localizes to micrometastatic ovarian carcinoma deposits within the peritoneal cavity. Dose estimates to microscopic lesions ranged from 14.7 to 81.4 cGy/mCi injected. Based on conventional radiobiology, these doses per mCi injected would be therapeutic in patients with minimal residual disease.

REFERENCES

1. XXX, XXX (Chapter 13). *In:* Cancer of the Ovary. *Editors:* Markman M. and Hoskins, W.J. Raven Press, New York, pp. xxx-xxx, 1993.

2. Rubin, S.C., Hoskins, W.J., Hakes, T.B., Markman, M., Cain, J.M. and Lewis, J.L.Jr. Recurrence after negative second-look laparotomy for ovarian cancer: Analysis of risk factors. Amer. J. Obstet. Gyn. 159:1094-1098, 1988.

3. Granowska, M., Shepard, J., Britton, K.E., Ward, B., Mather, S., Taylor-Papadimitriou, J., Epenetos, A.A., Carroll, M.J., Nimmon, C.C., Hawkins, L.A., et al.. Ovarian cancer: Diagnosis using ^{123}I monoclonal antibody in comparison with surgical findings. Nuclear Med. Commun. 5:485-499, 1984.

4. Epenetos, A., Shephard, J.H., Britton, K.E.,, Mather, S., Taylor-Papadimitriou, J., Granowska, M., Durbin, H., Nimmon, C.C., Hawkins, L.R., Malpas, J.S. and Bodmer, W.F. ^{123}I radioiodinated antibody imaging of occult ovarian cancer. Cancer 55:984-987, 1985.

5. Pateisky, N., Philipp, K., Skodler, W.D., Czerwenka, K., Hamilton, G. and Burchell, J. Radioimmunodetection in patients with suspected ovarian cancer. J. Nucl. Med. 26:1369-1376, 1985.

6. Chatal, J.F., Fumoleau, P., Saccavini, J.C., Thedrez, P., Curtet, C., Biano-Arco, A., Chetanneau, A., Peltier, P., Kremer, M. and Guillard, Y. Immunoscintigraphy of recurrences of gynecologic carcinomas. J. Nucl. Med. 28:1807-1819, 1987.

7. Stewart, J.S.W., Hird, V., Snook, D., Sullivan, M., Hooker, G., Coutenay-Luck, N., Sivolapenko, G., Griffiths., M., Myers, M.J., Lambert, H.E., Munro, A.J. and Epenetos, A.A. Intraperitoneal radioimmunotherapy for ovarian cancer: Pharmacokinetics, toxicity, and efficacy of I-131-labeled monoclonal antibodies. Int. J. Radiation Oncology Biol. Phys. 16:405-413, 1989.

8. Rubin, S.C., Kostakoglu, L., Divgi, C., Federici, M.G., Finstad, C.L., Lloyd, K.O., Larson, S.M. and Hoskins, W.J. Biodistribution and intraoperative evaluation of radiolabeled monoclonal antibody MX35 in patients with epithelial ovarian cancer.

Gynecol. Oncol. 51:61-66, 1993

9. Buist, M.R., Kenemans, P., Molthoff, C.J.M., Roos, J.C., Den Hollander, W., Brinkhuis, M. and Baak, J.P.A. Tumor uptake of intravenously administered radiolabeled antibodies in ovarian carcinoma patients in relation to antigen expression and other tumor characteristics. Int. J. Cancer 64:92-98, 1995.

10. Muto, M.G. and Kassis, A.I. Monoclonal antibodies used in the detection and treatment of epithelial ovarian cancer. Cancer 76:2016-2027, 1995.

11. Bradwell, A.R., Fairweather, D.S., Dykes, P.W., Keeling, A., Vaughan, A. and Taylor. J. Limiting factors in the localization of tumours with radiolabelled antibodies. Immunology Today 6:163-170, 1985.

12. Daghighian, F., Mazziotta, J.C., Hoffman, E.J., Shenderov, P., Eshaghian, B., Siegel, S. and Phelps, M.E. Intraoperative beta probe: a device for detecting tissue labeled with positron or electron emitting isotopes during surgery. Med. Phys. 21:153-157, 1994.

13. Mattes, M.J., Look, K., Furukawa, K., Pierce, V.K., Old L.J., Lewis, J.LJr. and Lloyd, K.O. Mouse monoclonal antibodies to human epithelial differentiation antigen expressed on the surface of ovarian carcinoma ascites cells. Cancer Res. 47:6741-6750, 1987.

14-15. Mattes, M.J., Lloyd, K.O. and Lewis, J.L.Jr. Binding parameters of monoclonal antibodies reacting with ovarian carcinoma ascites cells. Cancer Immunol. Immunother. 28:199-207, 1989.

16. Rubin, S.C., Finstad, C.L., Hoskins, W.J., Federici, M.G., Lloyd, K.O. and Lewis, J.L.Jr. A longitudinal study of antigen expression in epithelial ovarian cancer. Gynecol. Oncol. 34:389-394, 1989.

17. Nguyen, Q. and Heffelfinger, D.M. Imaging and quantitation of chemiluminescence using photoexcitable storage phosphor screen. Anal. Biochem. 226:59-67, 1995.

18. Humm, J.L., Chin, L.M., Lanza, R.C., Schaeffer, C.M., Speidel, M. and Greene, R.E. Digital Imaging for Autoradiography. Chapter 14. *In:* Computer Digital Radiography in Clinical Practice. *Editors:* Greene, R.E. and Oestmann, J.W. Thieme Medical Publishers, Inc., New York, pp. 167-172, 1992.

19. Kostakoglu, L., Rubin, S.C., Federici, M., Finstad, C.L., Larson, S.M., Hoskins, W.J. and Lloyd, K.O. A comparative study of intact and F(ab')2 fragments of monoclonal antibody MX35 in a xenograft model. J. Nucl. Med. 34:216P, 1993.

20. Hnatowich, D.J., Gionet, M., Rusckowski, M., Siebecker, D.A., Roche, J., Shealy, D., Mattis, J.A., Wilson, J., McGann, J., Hunter, R.E., Griffin, T. and Doherty, P.W. Pharmacokinetics of ^{111}In-labeled OC-125 antibody in cancer patients compared with the 19-9 antibody. Cancer Res. 47:6111-6117, 1987.

21. Breitz, H.B., Weiden, P.L., Vanderheyden, J.L., Appelbaum, J.W., Bjorn, M.J., Fer, M.F., Wolf, S.B., Ratliff, B.A., Seiler, C.A., Foisie, D.C., Fisher, D.R., Schroff, R.W., Fritzberg, A.R. and Abrams, P.G. Clinical experience with rhenium-186-labeled monoclonal antibodies for radioimmunotherapy: Results of phase I trials. J. Nucl. Med. 33:1099-1112, 1992.

22. Loevinger, R., Budinger, T.F. and Watson, E.E. MIRD Primer for Absorbed Dose Calculations. The Society of Nuclear Medicine, Inc., New York, 1991.

23. Humm, J.L. Dosimetric aspects of radiolabelled antibodies for tumor therapy. J. Nucl. Med. 27:1490-1497, 1986.

24. Goddu, S.M., Rao, D.V. and Howells, R.W. Multicellular dosimetry for micrometastases. Dependence of self-dose versus cross-dose to cell nuclei on type and energy of radiation and subcellular distribution of radionuclides. J. Nucl. Med. 35:521-530, 1994.

25. Griffith, M.H., Yorke, E.D., Wessels, B.W., De Nardo, G.L. and Neacy, W.P. Direct dose confirmation of quantitative autoradiography with micro-TLD measurements for radioimmunotherapy. J. Nucl. Med. 29:1795-1809, 1988.

26. Daghighian, F., Pentlow, K.S., Larson, S.M., Graham, M.C., DiResta, G.R., Yeh, S.D., Macapinlac, H., Finn, R.D., Arbit, E. and Cheung, N.K. Development of a method to measure kinetics of radiolabelled monoclonal antibody in human tumour with applications to microdosimetry: Positron emission tomography studies of iodine-124 labelled 3F8 monoclonal antibody in glioma. Eur. J. Nucl. Med. 20:402-409, 1993.

27. Rubin, S.C., Kairemo, K.J.A., Brownell, A.L., Daghighian, F., Federici, M.G., Pentlow, K.S., Finn, R.D., Lambrecht, R.M., Hoskins, W.J., Lewis, J.L.Jr. and Larson, S.M. High-resolution positron emission tomography of human ovarian cancer in nude rats using ^{124}I-labeled monoclonal antibodies. Gynecol. Oncol. 48:61-67, 1993.

28. Humm, J.L., Macklis, R.M., Lu, X.Q., Yang, Y., Bump, K., Beresford, B. and Chin, L.M. Spacial accuracy and cellular dose estimates obtained from 3D reconstructed serial tissue autoradiographs. Phys. Med. Biol. 40:163-180, 1995.

29. Schneider-Gadicke, E., Humm, J.L., Lau, C.C., Macklis, R.M., Bastert, G. and Knapp, R.C. Analysis of cytotoxicity of ^{131}I-labeled OC125 F(ab')2 on human epithelial ovarian cancer cell lines. Radiother. Oncol. 23:150-159, 1992.

Table 1. Summary of 35 Biopsied Specimens Analyzed from 6 Patients with Epithelial Ovarian Cancer[a]

Case #	Diagnosis	Number of biopsies from normal tissue	Number of biopsies with tumor cells	Tumor cells per biopsy (%)	Tumor cells immunostained (%) and intensity[b]
1	Endometrioid	3	6	< 20% clusters	90% ++
2	Serous	3	1	> 80% clusters	100% +++
3	Endometrioid with Clear Cell features	2	2	< 20% clusters & individual cells	75% + to ++
4	Endometrioid	2	1	> 50% clusters	100% +++
5	Carcinoma (poorly differentiated)	6	1	< 10% clusters	100% +++
6	Endometrioid (peritoneal)	3	5	< 10% clusters	100% +++

a: Tumor and normal tissue biopsied specimens were examined from all 6 cases by well scintillation counting and film autoradiography; biopsies from cases 2 - 6 were analyzed by storage phosphor screen autoradiography.

b: Frozen tissue sections by biopsied specimens were analyzed by indirect immunoperoxidase analysis: +++, strong; + to ++, weak and variable intensity.

Table 2. Percentage of Injected Dose per Gram (%ID/g) of Tumor, Normal Tissue Specimens and Blood for Radiolabelled mAb MX35 F(ab')2 by Well Scintillation Counting of Whole Biopsies.[a]

		%ID/g for ^{131}I-mAb MX35 F(ab')2			%ID/g for ^{125}I-mAb MX35 F(ab')2						
Case #	Time of Surgery[b]	Tumor (x 10^{-3})	Normal tissue (x 10^{-3}) *[fat]*	Blood (x 10^{-3})	Tumor (x 10^{-3})	Normal tissue (x 10^{-3}) *[fat]*	Blood (x 10^{-3})	T:Blood ^{131}I	T:Blood ^{125}I	T:Fat ^{131}I	T:Fat ^{125}I
1	4 day	1.35 - 3.18	0.35 - 1.26 *[0.35]*	4.82	0.82 - 1.83	0.21 - 0.75 *[0.21]*	2.86	0.28 - 0.70	0.29 - 0.64	3.82 - 8.96	3.92 - 8.78
2	5 day	8.71	0.54 - 1.71 *[0.54]*	3.17	5.89	0.34 - 1.02 *[0.34]*	2.05	2.75	2.89	18.21	17.42
3	4 day	0.52 - 1.33	0.49 - 0.53 *[0.53]*	3.41	0.29 - 0.74	0.28 - 0.32 *[0.32]*	1.95	0.15 - 0.39	0.15 - 0.38	0.87 - 2.49	0.88 - 2.28
4	1 day	22.35	1.27 - 7.27 *[1.26]*	4.42	21.09	1.13 - 6.90 *[1.13]*	3.76	5.05	5.61	17.69	18.73
5	5 day	7.98	0.22 - 1.72 *[0.22]*	2.14	6.36	0.16 - 1.33 *[0.16]*	1.63	3.73	3.91	35.89	38.96
6	4 day	0.83 - 1.30	0.52 - 0.70 *[0.59]*	2.25	0.65 - 1.10	0.38 - 0.58 *[0.47]*	1.75	0.37 - 0.58	0.37 - 0.63	1.40 - 2.20	1.40 - 2.36

a: Two milligrams of mAb MX35 F(ab') were labeled with ^{131}I and ^{125}I (cases 1 - 5). In case 6, radiolabeled mAb was mixed with 8 mg of cold mAb MX35 F(ab')2.
b: Monoclonal antibody was delivered by intravenous route (cases 1 - 3, 6) or by intrperitoneal route (cases 4 and 5) between 1 to 5 days prior to surgery.

Table 3. Summary of Dosimetry Results. Comparison of %ID/g by Well Scintillation Counts Versus Storage Phosphor Screens.

Case	Location of Tumor Specimen Analyzed	%ID/gr (well)	%ID/gr (phosphor)	(cGy) Dose/mCi Injected[a] (well)	(cGy) Dose/mCi Injected[b,c] (phosphor)
1	Omentum	3.18×10^{-3}	na	192.3	na
2	R Diaphragm	5.89×10^{-3}	16.2×10^{-3}	70.1	52.5
3	Omentum	1.33×10^{-3}	13.2×10^{-3}	12.6	18.9
4	Cul de sac	22.35×10^{-3}	358.0×10^{-3}	10.9	26.6
5	Para aortic LN	7.98×10^{-3}	18.0×10^{-3}	106.3	81.4
6	Fallopian tube	1.30×10^{-3}	10.2×10^{-3}	7.7	14.7

a: The dose estimates for the well scintillation counter assume a uniform distribution of activity.

b: The dose estimates from the storage phosphor screen take into account the heterogeneity of the radiolabeled antibody distribution and the associated absorbed fraction of local energy deposition. For a 100 µm tumor foci the absorbed energy is 0.17 MeV.

c: If a single administration of 100 mCi is dose limiting (bone marrow toxicity), then 1470 to 8140 cGy could be delivered to patients. In vitro studies on the radiosensitivity of ovarian carcinoma cell lines, OVCAR-3 and OVCA-433, have demonstrated that a radiation dose of approximately 1100 cGy is required to reduce the fraction of all surviving cells to 0.001 (ie., 3 logs cell kill).

FIGURE LEGENDS

5. Tumor biopsy from patient 2 with a serous ovarian carcinoma following administration of radiolabeled mAb MX35 F(ab')2 (5 days earlier) by intravenous route. Indirect immunoperoxidase staining with mAb MX35 at high power (A) and low power (B). Adjacent tumor sections of digitized film image at low power (C) and storage phosphor screen image at low power (D). Note the strong immunoperoxidase staining of tumor cell clusters and co-localization of radiolabeled mAb to the tumor.

6. Para aortic lymph node from patient 5 with a poorly differentiated ovarian carcinoma following administration of radiolabeled mAb MX35 F(ab')2 (5 days earlier) by intraperitoneal route. Indirect immunoperoxidase staining with mAb MX35 at high power (A) and low power (B). Adjacent tumor sections of digitized film image (saturated) at low power (C) and storage phosphor screen image at low power (D). Note the distribution of immunostained tumor cells between the hematopoietic cells and stromal tissue and localization of radiolabeled mAb to the tumor cell area specifically.

7. Tumor cell clusters in ovary (upper panel) and Fallopian tube (lower panel) from patient 6 with an endometrioid carcinoma of the peritoneum following administration of radiolabeled mAb MX35 F(ab')2 (4 days earlier) by intravenous route. Indirect immunoperoxidase staining with mAb MX35 at high power (A, E) and low power (B, F). Adjacent tumor sections of digitized film images at low power (C, G) and storage phosphor screen images at low power (D, H). Note that both the Fallopian tube epithelium and tumor cell clusters show strong immunoperoxidase staining, however, the radiolabeled mAb localizes to the tumor cell clusters on the film and phosphor screen images and not the adjacent normal tube epithelium.

8. Determination of specific activity of radionuclide within tumor specimens from patient 4 with an endometrioid ovarian carcinoma following administration of radiolabeled mAb MX35 F(ab')2 (1 day earlier) by intraperitoneal route. Three line profiles were drawn to traverse regions of micrometastatic tumor foci which impregnate the regions of normal stromal tissue (left panels). The location of the line profiles in the tumor biopsy are illustrated (right panel).

What is claimed is:

1. An $F(ab')_2$ fragment of the monoclonal antibody MX35.

2. A fragment according to claim 1 labeled with a detectable marker.

3. A fragment according to claim 2, wherein the detectable marker is radioactive.

4. A fragment according to claim 1 conjugated to a therapeutic agent.

5. A fragment according to claim 4, wherein the therapeutic agent is radioactive.

6. A method of detecting human ovarian cancer in a subject which comprises obtaining a suitable sample from the subject, contacting the suitable sample with an amount of the fragment of claim 2 effective to and under conditions permitting the fragment to form a complex with an antigen present on human ovarian cancer cells if present in the sample, and detecting any complexes so formed, thereby detecting human ovarian cancer in the subject.

7. A method according to claim 6, wherein detecting human ovarian cancer in the subject comprises detecting a micrometastatic tumor or micrometastatic tumors in the suitable sample obtained from the subject.

8. A method according to claim 7, wherein the suitable sample is obtained from the subject's peritoneal cavity.

9. A method according to claim 6, wherein detecting human ovarian cancer in the subject comprises detecting an epithelial ovarian carcinoma or epithelial ovarian carcinomas in the

subject.

10. A method of treating human ovarian cancer in a subject which comprises administering to the subject an amount of the fragment of claim 3 or claim 4 effective to treat human ovarian cancer.

11. A method according to claim 10, wherein the human ovarian cancer cells comprise a micrometastatic tumor or micrometastatic tumors.

12. A method according to claim 11, wherein the human ovarian cancer cells are located in the subject's peritoneal cavity.

13. a method according to claim 12, wherein administration comprises administering the fragment into the subject's peritoneal cavity.

14. A method of detecting human ovarian cancer in a subject which comprises administering to the subject an amount of the fragment of claim 2 effective to and under conditions permitting the fragment to specifically form a complex with an antigen present on human ovarian cancer cells if present within the subject, and detecting the detectable marker labelling the antibody so complexed.

15. A method according to claim 14, wherein detecting human ovarian cancer in the subject comprises detecting a micrometastatic tumor or micrometastatic tumors in the subject.

16. A method according to claim 14, wherein the micrometastatic tumor or micrometastatic tumors are in the subject's peritoneal cavity.

17. A method according to claim 16, wherein administration

comprises administering the fragment into the subject's peritoneal cavity.

FIG. 1

FIG. 2A

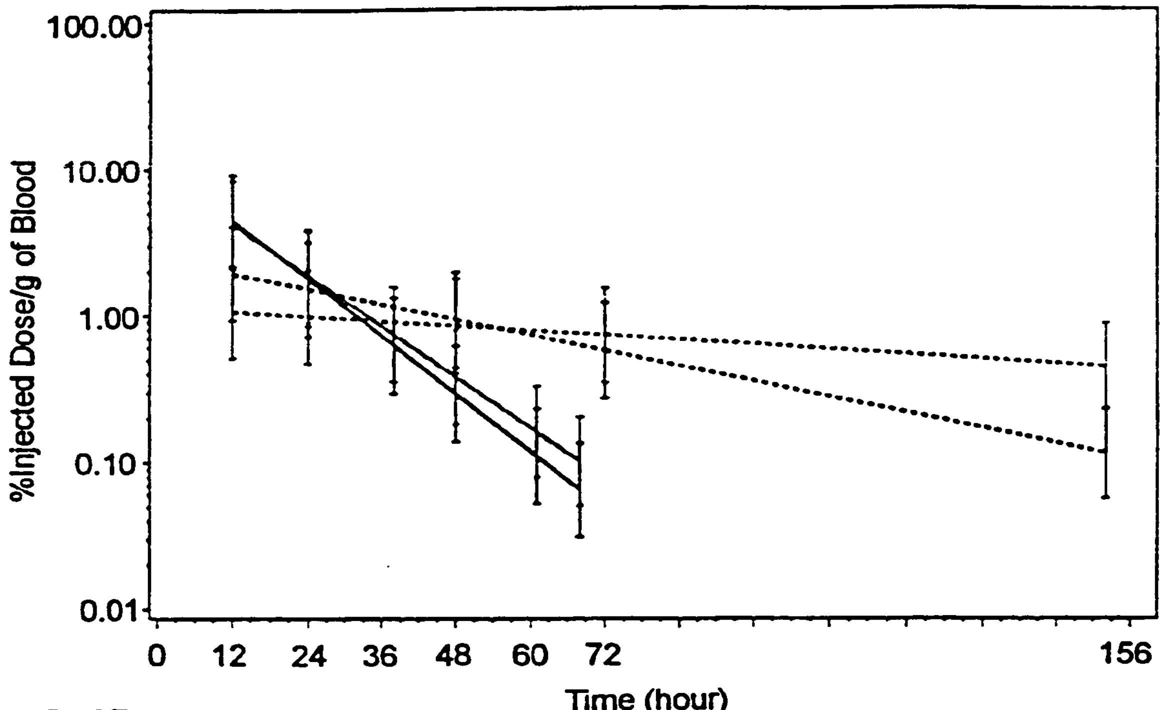

100.00
10.00
1.00
0.10
0.01
%Injected Dose/g of Blood
0 12 24 36 48 60 72 156
Time (hour)

FIG. 2B

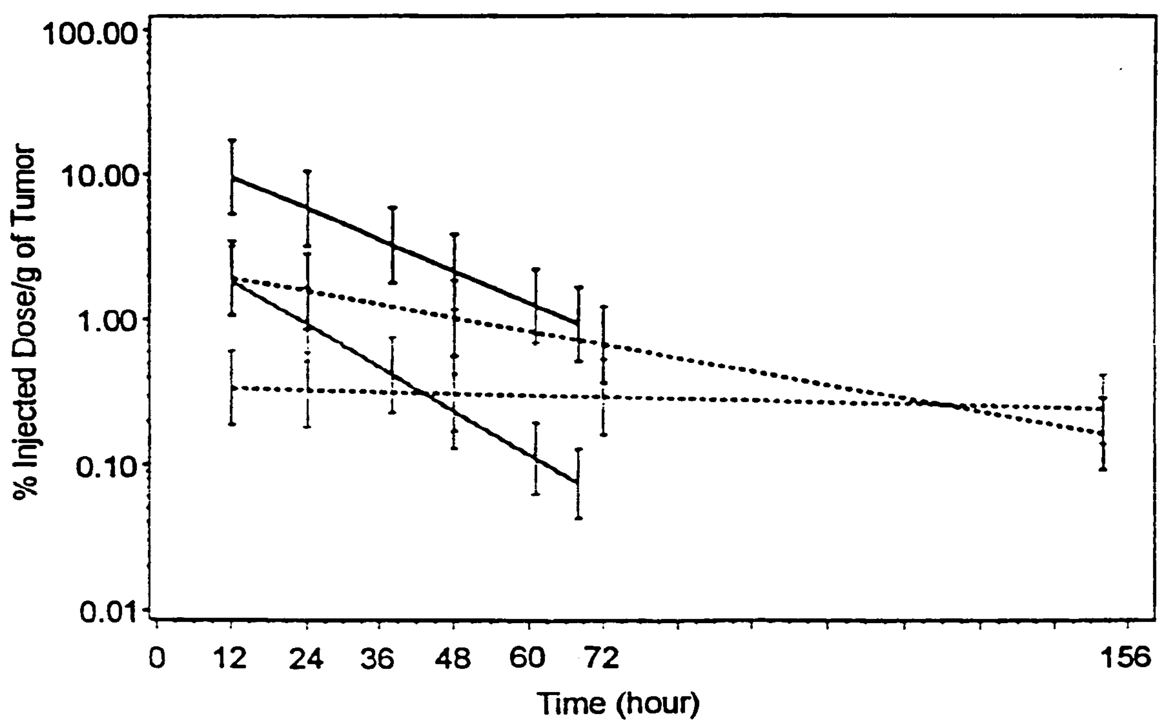

100.00
10.00
1.00
0.10
0.01
% Injected Dose/g of Tumor
0 12 24 36 48 60 72 156
Time (hour)

FIG. 3A

FIG. 3B

FIG. 3C

FIG. 3D

FIG. 3E

FIG. 3F

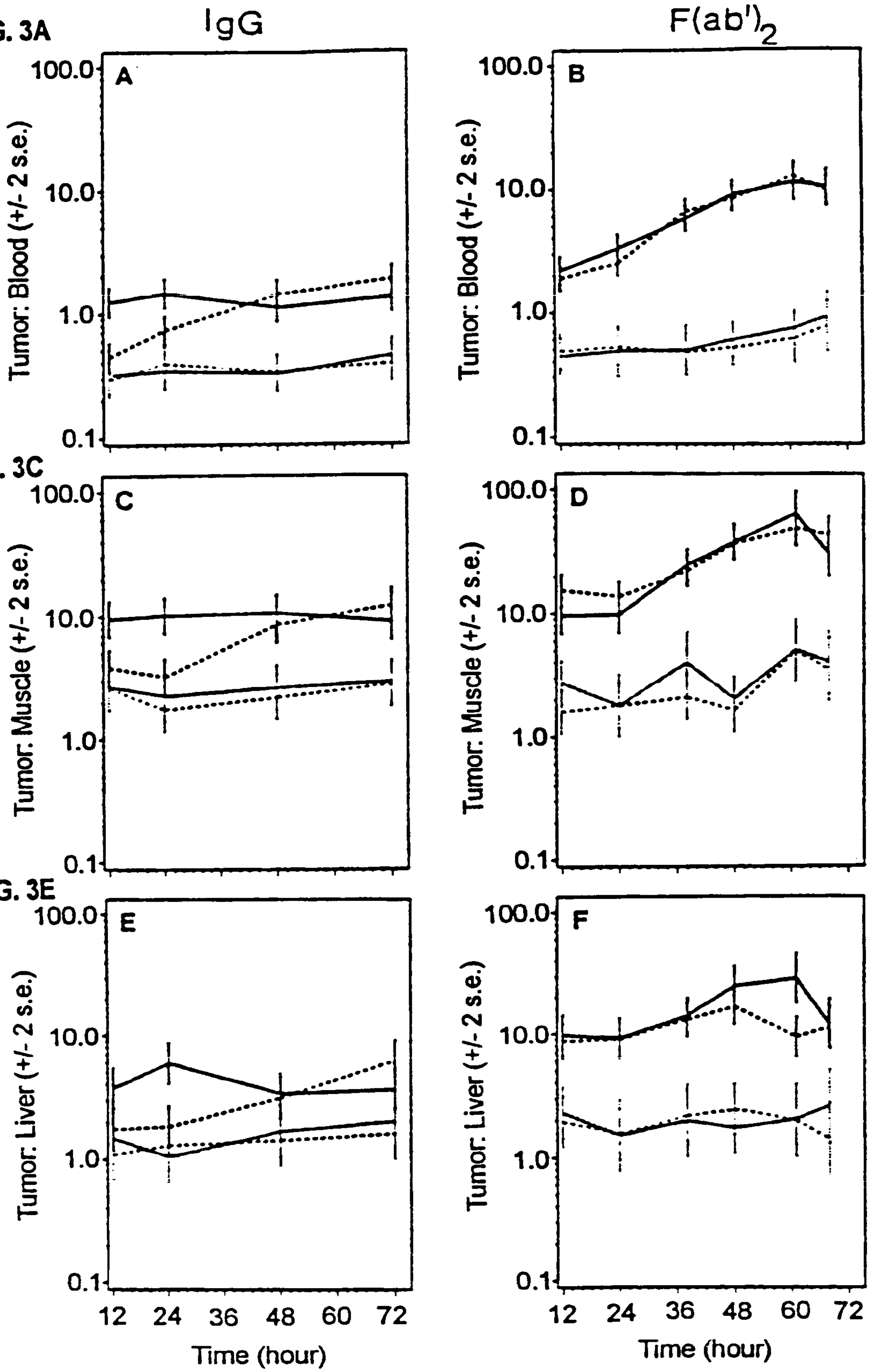


FIG. 4

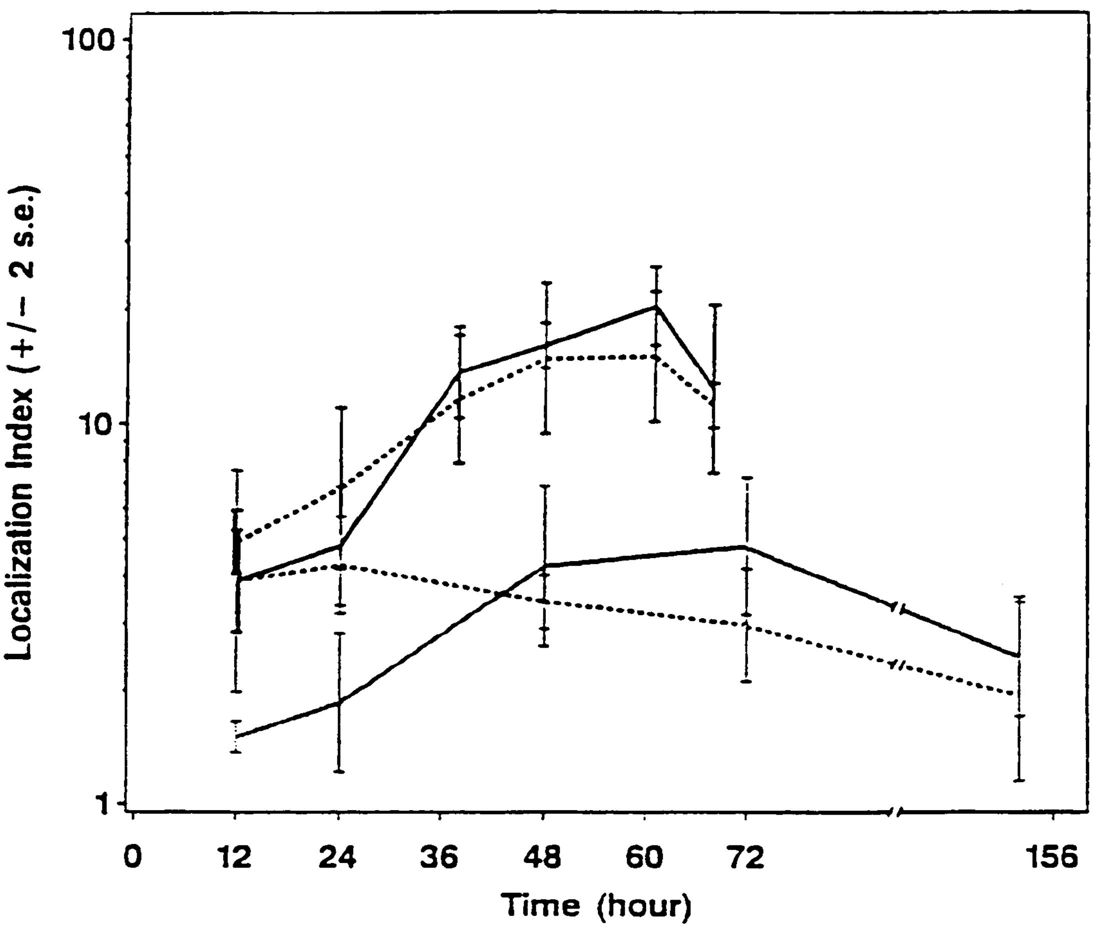

100
10
1
Localization Index (+/− 2 s.e.)
0
12
24
36
48
60
72
156
Time (hour)

FIG. 5A

Patient 2 - 2mg i.v.

Antibody stain
FIG. 5B

X-ray film
FIG. 5C

Phosphor screen
FIG. 5D

Patient 5 - 2mg i.p.

FIG. 6A

Antibody stain

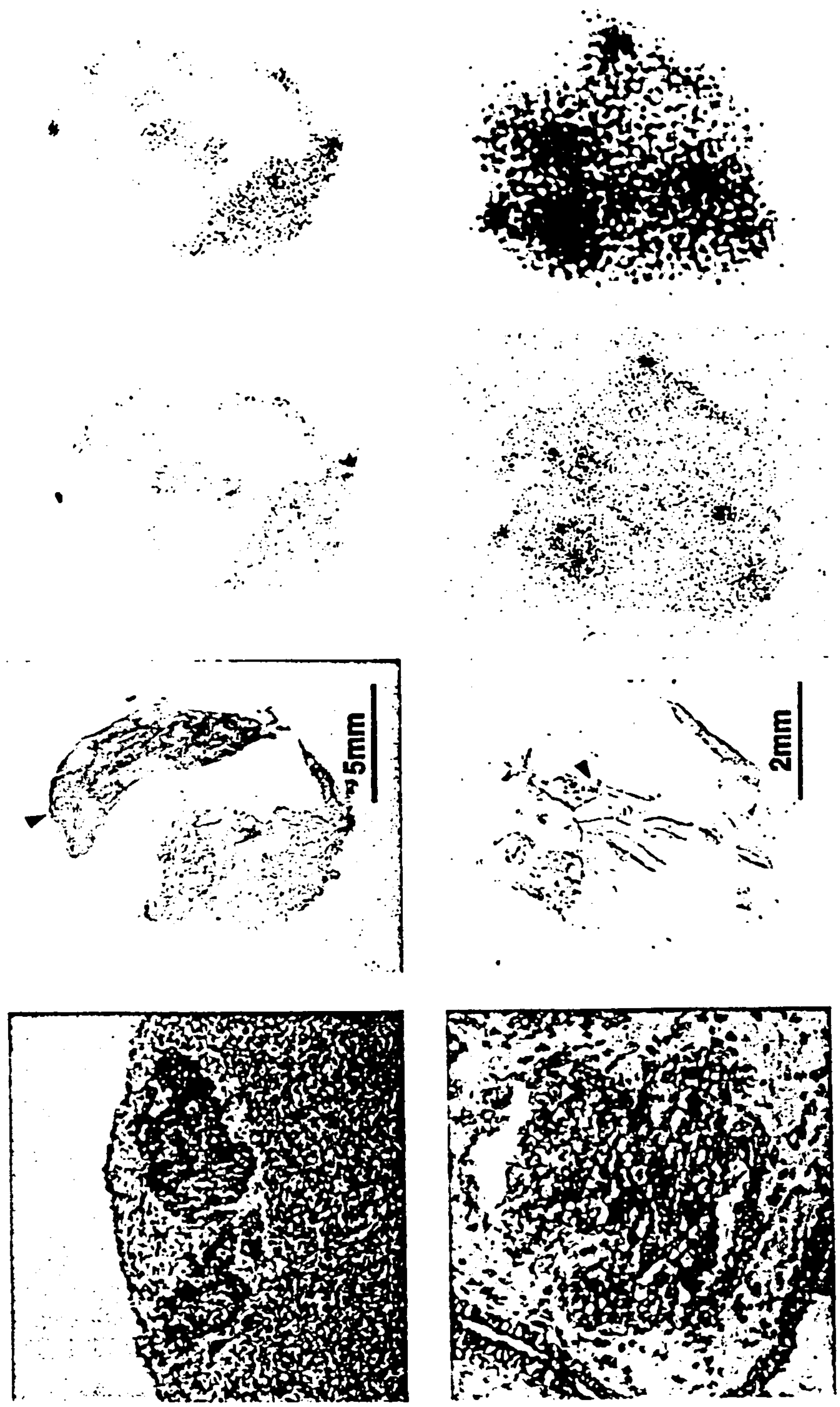
FIG. 6B

X-ray film

FIG. 6C

Phosphor image

FIG. 6D



Patient 6 - 10mg i.v.

FIG. 7A FIG. 7B FIG. 7C FIG. 7D

Antibody stain X-ray film Phosphor Image

FIG. 7E FIG. 7F FIG. 7G FIG. 7H

FIG. 8A

FIG. 8B

FIG. 8C

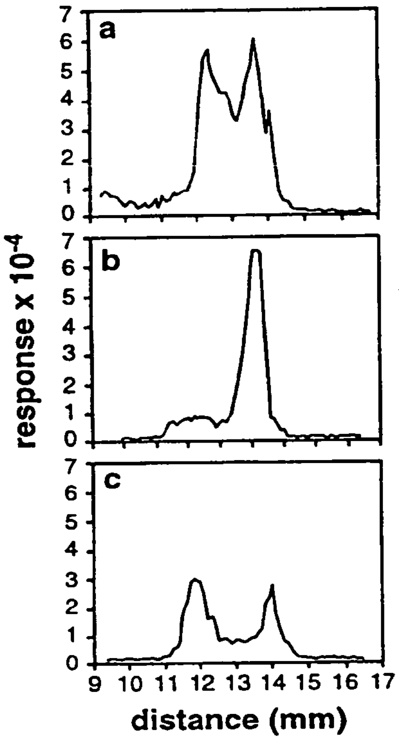


FIG. 8D

Phosphor screen image

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09819

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 51/10, 49/00, 39/395; C07K 16/30; C12N 5/12; G01N 33/53
US CL :424/1.49, 9, 156.1; 530/388.85, 391.3, 391.7; 435/7.23, 240.27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.49, 9, 156.1; 530/388.85, 391.3, 391.7; 435/7.23, 240.27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Immunology and Immunotherapy, Volume 28, issued 1989, Mattes et al., "Binding parameters of monoclonal antibodies reacting with ovarian carcinoma ascites cells", pages 199-207, see entire document.	1-17
Y	Cancer Research, Volume 47, issued 15 December 1987, Mattes et al., "Mouse monoclonal antibodies to human epithelial differentiation antigens expressed on the surface of ovarian carcinoma ascites cells", pages 6741-6750, see entire document.	1-17
Y	Medical Physics, Volume 20, Number 2, issued March/April 1993, Buchsbaum et al., "Experimental radioimmunotherapy", pages 551-67, especially page 555.	1-17

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
19 JULY 1996	02 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PAULA HUTZELL Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09819

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	S. Broder, "Molecular foundations of oncology", published 1991 by Williams & Wilkins, pages 95-134, see entire document.	1-17